REMARKS

Reconsideration of the Office Action mailed July 2, 2004, (hereinafter "instant Office Action"), entry of the foregoing amendments and withdrawal of the rejection of claims 1-35, 37-40 and 44-88, are respectfully requested.

In the instant Office Action, claims 1-35, 37-40 and 44-88 are listed as pending, and claims 1-35, 37-40 and 44-88 are listed as rejected.

Applicants thank the Examiner for his time during the telephonic interview conducted on December 2, 2004. Applicants appreciate the opportunity to speak with the Examiner.

Claims 1, 6-14, 16, 18-20, 22, 24, 31, 32, 52 and 65 have been amended to correct typographical errors.

Applicants observe that the Examiner has not mentioned the rejection of claims 1-40 and 44-88 under 35 U.S.C. §112, second paragraph, for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention with respect to the terms "substituted" and "heterocyclic", and therefore, Applicants presume that the arguments and amendments submitted in the Preliminary Amendment filed concurrently with the Request for Continued Examination filed on May 17, 2004 was persuasive and the rejections have been withdrawn. Applicants respectfully request that the Examiner advise Applicants if this is not correct.

The Examiner has rejected claims 1-35, 37-40 and 44-88 under 35 U.S.C.§112, second paragraph, for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Applicants respectfully traverse this rejection. Applicants' response to the Examiner's enumerated points are numbered accordingly to track the Examiner's points.

i) With respect to the terms Z^{110}, Z^{111}, Z^{105}, Z^{101} and Z^{200} the Examiner alleges that the term "alkyl" is missing following "(C_1-C_6)". As stated in MPEP 2173.01:

> A fundamental principle contained in 35 USC 112, second paragraph is that applicants are their own lexicographers. They can define in the claims what they regard as their invention essentially in whatever terms they choose so long as any special meaning assigned to a term is clearly set forth in the specification

Applicants respectfully direct the Examiner's attention to page 54, lines 19-21, wherein Applicants state in the specification as originally filed "As used herein, aliphatic groups or notations such as "(C_0-C_6)" include straight chained, branched or cyclic hydrocarbons which are completely saturated or which contain one or more units of unsaturation." It is understood by one of ordinary skill in the art that "(C_1-C_6)", as defined on page 54, lines 19-21, can mean alkyl, alkenyl or alkynyl, as well as cycloalkyl or cycloalkenyl and should not be limited to merely "alkyl". Thus, it is clear what is meant by the term "(C_1-C_6)" and therefore it is not necessary to add "alkyl" because that would limit the definition of "(C_1-C_6)" versus its true meaning as defined in the specification.

ii) The Examiner points out that claim 46 depends on cancelled claim 36, claim 48 depends on claim 46, claim 49 depends on claim 48 and claim 50 depends on claim 46. Applicants have cancelled claims 46, 48, 49 and 50 without waiver or prejudice to Applicants' right to pursue the cancelled subject matter in a continuation or divisional application.

Based upon the foregoing, the rejection of claims 1-35, 37-40 and 44-88 under 35 U.S.C.§112, second paragraph, for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention, is obviated and should be withdrawn.

The Examiner has rejected claims 33-35, 37-40, 44, 47 and 51 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make or use the invention. Applicants respectfully traverse this rejection.

On page 3 of the instant Office Action, the Examiner states that the specification fails to teach any benefit to be gained from inhibiting one or more protein kinase activity. On page 22, lines 7-20 of the instant specification Applicants state:

> In particular, compounds of this invention are useful as inhibitors of tyrosine kinases that are important in hyperproliferative diseases, especially in cancer and in the process of angiogenesis. For example, certain of these compounds are inhibitors of such receptor kinases as KDR, Flt-1, FGFR, PDGFR, c-Met, TIE-2 or IGF-1-R. Since certain of these compounds are anti-angiogenic, they are important substances for inhibiting the progression of disease states where angiogenesis is an important component. Certain compounds of the invention are effective as inhbitors of such serine/threonine kinases as PKCs, erk, MAP kinases, cdks, Plk-1 or Raf-1. These compounds are useful in the treatment of

> cancer, and hyperproliferative disorders. In addition, certain compounds are effective inhibitors of non-receptor kinases such as those of the Src (for example, Lck, blk and lyn), Tec, Csk, Jak, Map, Nik and Syk families. These compunds are useful in the treatment of cancer, hyperproliferative disorders and immunologic diseases.

Thus, Applicants have taught a benefit to inhibiting one or more protein kinase activities, that is, treatment of cancer, hyperproliferative disorders and immunological diseases.

Applicants respectfully submit that the inquiry that has to be answered to meet the enablement requirement is best phrased as follows: "If one were to use a compound of formula I to inhibit one or more protein kinase activities, how would one do it?" This is a very different question from the question that the Examiner is asking, which is best phrased as follows: "If one were to use a compound of formula I to inhibit one or more protein kinase activities, would it work?" Applicants respectfully submit that it is the first phrased question that has to be answered to meet the enablement requirement of 35 U.S.C. §112, first paragraph. Applicants submit that Applicants have answered the question by detailing: (1) how one of ordinary skill in the art would obtain a compound of formula I; (2) how one of ordinary skill in the art would formulate a compound of formula I; and (3) how one of ordinary skill in the art would administer such a formulation of a compound of formula I to inhibit one or more protein kinase activities.

The Examiner alleges that pharmacological activity in general is a very unpredictable area. The Examiner states "...'the scope of enablement obviously varies inversely with the degree of unpredictability of the factors involved.' See *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970)." Applicants respectfully point out that *In re Fisher* also found that:

> As long as the specification discloses at least one method for making and using the claimed invention that bears reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. 112 is satisfied.

Applicants have taught how to make and how to use the instant invention and provided assays to measure protein kinase inhibition on pages 76 to 88 of the instant specification.

The Examiner states "[t]he notion that simply inhibiting one or more protein kinase activity will enable a whole list of unrelated disorders is not substantiated". Applicants attach a copy of the review article "Tyrosine kinases in disease: overview of kinase inhibitors as therapeutic agents and current drugs in clinical trials", Strawn et al., *Expert Opinion on*

Investigational Drugs, (1998) 7(4):553-573, as Exhibit A, which addresses the Examiner's concern regarding the list of disorders enumerated in the claims.

The fact that the Examiner has cited *In re Ruskin* 148 USPQ 221 and *Ex parte Jovanovics* 211 USPQ 907) and states that the Examiner has the authority to require evidence that tests relied on are reasonably predictive of in vivo activity by those skilled in the art is further proof that the Examiner has confused lack of utility with lack of enablement. Neither *In re Ruskin* or *Ex parte Jovanovics* is on point. In both cases, the claims under appeal were rejected under 35 U.S.C. §101, for lack of utility, not 35 U.S.C. §112, first paragraph, for lack of enablement.

The Examiner has confused the requirement for utility versus the requirement for enabling how to make and how to use. The Examiner's rejection is not consistent with the Utility Guidelines issued by the U.S.P.T.O., which state "[When] applicant discloses a specific biological activity and reasonably correlates that activity to a disease condition...[it] would be sufficient to identify a specific utility for the compound". The Examiner's rejection seeks proof that a compound of formula I is effective in inhibiting one or more protein kinase activities. However, such evidence is not required for patentability. Applicants respectfully direct the Examiner's attention to M.P.E.P. 2107.03 V, which states:

> Thus, while an applicant may on occasion need to provide evidence to show that an invention will work as claimed, it is improper for Office personnel to request evidence of safety in the treatment of humans, or regarding the degree of effectiveness. See *In re Sichert*, 566 F.2d 1154, 196 USPQ 209 (CCPA 1977); *In re Hartop*, 311 F.2d 249, 135 USPQ 419 (CCPA 1962); *In re Anthony*, 414 F.2d 1383, 162 USPQ 594 (CCPA 1969); *In re Watson*, 517 F.2d 465, 186 USPQ 11 (CCPA 1975); *In re Krimmel*, 292 F.2d 948, 130 USPQ 215 (CCPA 1961); *Ex parte Jovanovics*, 211 USPQ 907 (Bd. Pat. App. & Inter. 1981).

35 U.S.C. §101 is concerned with whether an invention is useful. 35 U.S.C. §112, first paragraph, on the other hand, is concerned with how to make and use the invention. The Examiner is also focusing on "the compound", not "the invention". Applicants have taught how to make and use the instant invention. Nevertheless, Applicants respectfully point out that the Utility Guidelines state the following:

> Courts have repeatedly found that the mere identification of a pharmacological activity of a compound that is relevant to an asserted

pharmacological use provides an "immediate benefit to the public" and thus satisfied the utility requirement. As the CCPA held in Nelson v. Bowler:

> Knowledge of the pharmacological activity of any compound is obviously beneficial to the public. It is inherently faster and easier to combat illnesses and alleviate symptoms when the medical profession is armed with an arsenal of chemicals having known pharmacological activities. Since it is crucial to provide researchers with an incentive to disclose pharmacological activities in as many compounds as possible, we conclude that adequate proof of any such activity constitutes a showing of practical utility.

Similarly, courts have found utility for therapeutic inventions despite the fact that an applicant is at a very early stage in the development of a pharmaceutical product or therapeutic regimen based on a claimed pharmacological or bioactive compound or composition.

Where an applicant has established utility for a species that falls within a[n] identified genus of compounds and presents a generic claim covering the genus, as a general matter, that claim should be treated as being sufficient under §101.

Thus, Applicants have enabled the instant invention, as well as satisfied all requirements for utility.

The Examiner states that:

> ...the Examiner has the authority to require evidence that tests relied on are reasonably predictive of in vivo activity by those skilled in the art. See for example, In re Ruskin, 148 USPQ 221: Ex parte Jovanics 211 USPQ 907 and M.P.E.P. 2164.05(e)."

With respect to the Examiner's citation of MPEP 2164.05(e), Applicants respectfully point out that there is no section (e) in MPEP 2164.05. Applicants presume that the Examiner is referring to MPEP 2164.05 which states "Applicant may submit factual affidavits under 37 CFR 1.132 or cite references to show what one skilled in the art knew at the time of the filing of the application." The *in vitro* model detailed on page 82 is a well-validated model of T-cell activation, also known as the mixed lymphocyte reaction. This is an *in vitro* model of direct T-cell recognition of allogeneic MHC molecules and is used as a predictive test of T-cell mediated transplant rejection, the in vivo model defined on page 84. This predictive connection is covered in detail in "Fundamental Immunology, 4th edition, 1999, Chapter 36, Transplatation

Immunology, by Hugh Auchincloss et al" and references cited therein. Applicants submit herewith, as Exhibit B, a copy of the above-cited chapter for the Examiner's convenience.

The Examiner states that "[t]he scope of uses embraced by these compounds are not remotely enabled based solely on instant compounds ability to inhibit one or more protein kinase activity". Applicants respectfully direct the Examiner's attention to page 55, lines 25 to page 56, line 23 wherein Applicants describe that compounds have antiangiogenic properties due at least in part to the inhibition of protein tyrosine kinases and then lists diseases in which the compounds can be used. As discussed above, Applicants have attached a copy of a review article (Exhibit A) which addresses the Examiner's concerns regarding the scope of uses.

With respect to enablement and utility, the Utility Guidelines state:

> "An applicant need only make one credible assertion of specific utility for the claimed invention to satisfy §101 and §112; additional statements of utility, even if not "credible" do not render the claimed invention lacking in utility.

The Examiner states that "[t]he how to use portion of the statute has not been addressed. This means that Applicants must teach the skilled practitioner, in this case a physician, how to treat a given subject". The Examiner cites *In re Lorenz and Wegler*, 13 USPQ 312 U.S. Court of Customs and Patent Appeals. In *In re Lorenz and Wegler*, the court found that the only attempt to comply with the use requirements of Section 112 was found in the appellants specification which stated:

> A characteristic feature of the novel phosphoric acid ester is their low toxic effects against warm-blooded animals, while at the same time they have a good effect against a very wide range of insects.

The court found that no one would know how to use one of the claimed compounds to kill even a single insect or whether the compounds were to be used as sprays, dusts or in another manner. With respect to the Examiner's comments that Applicants have not provided what is being treated by claim 33, Applicants respectfully direct the Examiner's attention to page 55, line 25 to page 56, line 23 of the instant specification wherein Applicants describe that the compounds of the invention have antiangiogenic properties due at least in part to the inhibition of protein tyrosine kinases, followed by a list of the diseases in which the compounds can be used.

With respect to the Examiner's comment that Applicants have not stated who the subject is in claim 33, claim 33 has been amended to add the phrase "in need thereof". Support for this

amendment can be found, *inter alia*, at page 59, line 11. The Examiner asks how one can identify a subject in need. As stated above, Applicants have at page 55, line 25 to page 56, line 23 of the instant specification listed disease states the compounds of the instant invention can be used against. Therefore, any patient presenting with any of the aforementioned disease states would be "a patient in need".

With respect to the Examiner's objection that Applicants have given no specific dose, given no specific dosing regimen and given no specific route of administration, Applicant respectfully direct the Examiner's attention to the Pharmaceutical Formulation section on pages 66-70 of the instant specification wherein Applicants describe routes of administration. Applicants particularly direct the Examiner's attention to page 72, lines 21-23 of the instant application wherein it states "The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician." Only the physician directing the care of a patient can determine the best course of treatment (route of administration, dosage) for a patient depending upon factors specific to each individual patient (weight, severity of disease). The specification clearly conveys to one skilled in the art what is being treated by claim 33, who the subject is, how one can identify said subject, and how dosage, dosing regimen and route of administration can be determined.

From this language it appears that the Examiner may be applying approval by the Food and Drug Administration as the standard for utility and/or enablement under the patent statutes. Applicants direct the Examiner's attention to M.P.E.P. 2107.02 V. which states:

> FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. *In re Brana* 51 F.3d 1560, 34 USPQ2d 1436 (Fed. Cir. 1995) (citing *Scott v. Finney*, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed. Cir. 1994)).

The foregoing passage clearly indicates that it is not necessary that a compound obtain approval from or be approvable by the Food and Drug Administration in order to meet the enablement requirement of 35 U.S.C. §112, first paragraph. The statute only requires an applicant to show how to make and how to use an invention. In the instant application, Applicants have shown how to make and use a compound of formula I or a pharmaceutically acceptable salt thereof for inhibiting one or more protein kinase activities.

If the Examiner's apparent question of "if one were to use a compound of formula I to inhibit one or more protein kinase activities, would it treat a disease?" was what had to be answered to satisfy 35 U.S.C. §112, first paragraph, then all pharmaceutical patent applications would have to include clinical efficacy data. This is not the case. In fact, the M.P.E.P. cautions against requiring such data. Applicants direct the Examiner's attention to M.P.E.P. 2107.02 IV, which states:

> Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials. There is no decisional law that requires an applicant to provide data from human clinical trials to establish utility for an invention related to treatment of human disorders (see *In re Isaacs*, 347 F.2d 89, 146 USPQ 193 (CCPA 1963); *In re Langer*, 503 F.2d 1380, 183 USPQ 288 (CCPA 1974)), even with respect to situations where no art-recognized animal models existed for the human disease encompassed by the claims.

The Examiner cites *In re Moureu and Chovin*, 145 USPQ 452, in which the U.S. Court of customs and Patent Appeals found that the application contained no disclosure of the utility and methods of using the claimed compounds other than statements that the compounds were found to possess highly useful pharmacological properties, substantial anti-tubercular activity and could be employed in veterinary medicine against five different conditions. The court also found that the appellants had not disclosed or suggested the manner of administration. The instant case differs in that Applicants have described routes of administration. Further, Applicants have provided assays which can be used to test the efficacy of the compounds with regard to kinase inhibition, not merely stated that the compounds of the instant invention are useful as kinase inhibitors. With respect to the court's finding that

> ...those skilled in the art who desire to use the products of the invention for medicinal purposes would find it necessary to engage in extensive experimentation to determine what would be the effective and safe manner of using the products as medicines for the suggested purposes and to determine the dosages to be avoided because lethal or ineffective

the amount of experimentation need to determine the effective and safe manner of using the products as medicines is routine in the pharmaceutical industry. Thus the experimentation required would not be considered extensive. Further, Applicants submit that safety is the province of the U.S. Food and Drug Administration, not the U.S. Patent and Trademark Office.

With respect to the Examiner's citation of *Brenner v. Manson*, 148 USPQ at 696, and the finding that

> ...it was not the intention of the statutes to require the Patent Office, the courts, or the public to play the sort of guessing game that might be involved if an applicant could satisfy the requirements of the statues by indicating the usefulness of a claimed compound in terms of possible use so general as to be meaningless...

Applicants respectfully point out that in the instant application Applicants have taught how to make and use the compounds of the invention as well as provided assays which can be used to test the effectiveness of each compound as a kinase inhibitor. Applicants have taught how to use the compounds of the instant invention in the Pharmaceutical Formulation section of the application on pages 66, line 10 through page 70, line 30. Applicants have taught that there are diseases which can be affected by inhibition of certain kinases at page 55, line 25 to page 56, line 24 and written claims drawn to using the instant compounds to treat said diseases. Applicants have provided a detailed explanation of how, using the compounds of the instant invention, the inhibition of protein kinase activity can be used in a therapeutic context.

Based upon the foregoing, the rejection of claims 33-35, 37-40, 44, 47 and 51 under 35 U.S.C. §112, first paragraph, is obviated and should be withdrawn.

The Examiner has rejected claims 1-32 under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention. Applicants respectfully traverse this rejection.

With respect to the Examiner's statement that "[o]nly one compound has been made. This does not give a reasonable assurance that all, or substantially all of the compounds that could be made are useful." Applicants respectfully point out that there is no requirement to provide working examples, much less submit data. As stated in M.P.E.P. 2164.02:

> Compliance with the enablement requirement of 35 U.S.C. 112, first paragraph does not turn on whether an example is disclosed....An applicant need not have actually reduced the invention to practice prior to filing

Therefore, the number of working examples contained in a specification is not relevant in determining whether an applicant has met the requirements of 35 U.S.C. §112, first paragraph.

What matters is whether the applicant has taught how to make and use the invention. In the instant case Applicants have satisfied 35 U.S.C. §112, first paragraph.

The Examiner states "The claims are not drawn in terms of a recognized genus but are directed to a more or less artificial selection of compounds." Applicants respectfully point out that the Examiner issued a Restriction Requirement in the instant case. Although Applicants traversed the Restriction Requirement, argued that the heterocycles of formulae 1-117 represented different embodiments of a single inventive concept, and pointed out the single, searchable unifying aspect, inhibition of kinase activity, that linked all of the heterocycles, the Examiner made the Restriction Requirement final. In compliance with the Examiner's requirement, Applicants elected a single species to be searched. Thus, Applicants submit that the genus to which the Examiner has expanded his search is a genus created by the Examiner's Restriction Requirement.

The Examiner cites *In re Surrey* 151 USPQ 724, regarding the sufficiency of a disclosure for a Markush group and MPEP 2164.03 for enablement requirements in cases directed to structure-sensitive arts such as the instant pharmaceutical arts. In *In re Surrey*, the case involved the rejection under 35 USC §112, first paragraph, of a claim containing a Markush group. In *In re Surrey*, only a small, homogenous group of the compounds which had been tested exhibited the asserted psychomotor stimulatory and anticonvulsant properties. Based upon this, the court found that the Markush group was not supported. However, the court also stated "It is manifestly impracticable for an applicant who discloses a generic invention to give an example of every species falling within it, or even to name every such species. It is sufficient if the disclosure teaches those skilled in the art what the invention is and how to practice it." Applicants have taught how to make and use the instant invention.

The Examiner further states "There is no reason why a claim drawn in this way should not be limited to those compounds which are shown to be useful". The Court of Customs and Patent Appeals (CCPA) has stated that the specification need not contain a working example of every embodiment of the invention "if the invention is otherwise disclosed in such a manner that one skilled in the art would be able to practice it." In re Borkowski, 164 U.S.P.Q. 642, 645 (CCPA 1970). See United States v. Telectronics, Inc., 8 U.S.P.Q. 2d 1217 (Fed. Cir. 1988). Applicants have disclosed the instant invention in a manner in which one skilled in the art can practice it as

well as disclosed a utility it. Therefore, there is no reason to further limit the claims, as they are drawn to useful compounds.

Based upon the foregoing, the rejection of claims 1-32 under 35 U.S.C. §112, first paragraph, is obviated and should be withdrawn.

The Examiner has rejected claims 1-40 and 44-88 under 35 U.S.C. §103(a) as allegedly being unpatentable over Altmann et al. (WO 97/49706). Applicants respectfully traverse this rejection. Applicants maintain the arguments presented in the Replies filed August 26, 2002, November 26, 2002, July 26, 2003, March 15, 2004 and May 17, 2004.

The Examiner alleges that "the reference teaches a generic group of substituted 7-amino-pyrrolo[3,2-d]pyrimidine derivatives which embraces applicants' claimed compounds". The Examiner points to Example 72 on page 25. Applicants respectfully point out that Example 72 appears on page 35 and have referenced that example because it corresponds with the Examiner's description. The Examiner states "...the reference teaches the equivalence of substituted aryl groups (listed on pages 3-4) as the most especially preferred compounds (see page 8, 3rd paragraph)." Applicants respectfully point out that Altmann et al. teaches "[a]ryl is, for example, phenyl or naphthyl..." on page 2. Nowhere does Altmann et al. teach or suggest pyrrolyl, pyrazolyl, pyridinyl, imidazolyl, pyrazolinyl or pyrimidinyl at the position Applicants designate Ring 1, all of which Applicants teach in this position. Altmann et al. does not suggest Applicants' invention as a whole.

As Applicants have argued in the Replies filed previously, an invention is to be considered as a whole. The claimed invention may not be dissected into discrete elements to be analyzed in isolation, but must be considered as a whole. See, e.g. W.L. Gore & Assoc. Inc. v. Garlock, Inc. 721 F.2d 1540, 1548, 220 USPQ 303, 309 (Fed. Cir. 1983)); Jones v. Hardy, 727 F.2d 1524, 1530, 220 USPQ 1021, 1026 (Fed. Cir. 1983). The Examiner has not shown how Altmann et al. render obvious the entire genus of Applicants' claim.

The Examiner further states "A claim is unpatentable if only one embodiment within its scope is unpatentable." However, in In re Jones, the CCPA found differently. In In re Jones (958 F2d 347, 350, 21 USPQ2d 1941, 1943 (Fed. Cir. 1992) the Examiner rejected a claim to the 2-(2'-aminoethoxy)ethanol salt of dicamba as obvious over U.S. Patent No. 3,013,054 ("Richter") and 86 Chem. Abstracts No. 437711a ("Wideman"). Richter disclosed a genus which admittedly encompassed the claimed salt but did not specifically disclose the claimed 2-

(2'-aminoethoxy)ethanol salt. Richter did not teach the amine needed to prepare this salt, but Wideman did. Wideman, however, was directed to the use of that amine in the preparation of surfactants for shampoos, bath preparations, and emulsifiers. The Board of Appeals upheld the Examiner's obviousness rejection and also noted structural similarity between the claimed salt and the disclosed genus in the prior art. The CCPA, however, found that a disclosure of a chemical genus does not render obvious a species that happens to fall within it. The CCPA also found that there was not close structural similarity because the claimed salt was a primary amine with an ether linkage, whereas the diethanolamino salt disclosed by Richter is a secondary amine without an ether linkage. Thus, the CCPA concluded that the Examiner had failed to establish a *prima facie* case of obviousness.

Although In re Jones deals with the species-genus relationship with respect to obviousness, it is applicable to the instant case. In In re Jones a genus was disclosed which encompassed the Appellants' claimed compound. In the instant case the reference, Altmann et al. describes a genus which overlaps with Applicants' claimed genus. Altmann et al.'s genus does not make Applicants' genus as a whole obvious. Altmann et al.'s genus does not encompass Applicants' species.

An invention is to be considered as a whole. In determining the differences between the prior art and the claims, the question under 35 U.S.C. §103 is not whether the differences themselves would have been obvious, but whether the claimed invention as a whole would have been obvious. Stratoflex, Inc. v. Aeroquip Corp., 713 F.2d 1530, 218 USPQ 871 (Fed. Cir. 1983); Schenck v. Nortron Corp.,713 F.2d 782, 218 USPQ 698 (Fed. Cir. 1983). The Examiner has not shown how Altmann et al. renders obvious the entire genus of Applicants' claim.

> In referring to Example 72 on page 35 of the reference, the Examiner states:
>
> One skilled in the art would be motivated to modify this prior art compound and arrive at the instant claims (where, for example, R_1 is phenyl, substituted at the 4-position by A-Z^{100}) because the reference teaches the equivalence of substituted aryl groups (listed on pages 3-4) as the most especially preferred compounds (see page 8, 3rd paragraph).

Applicants respectfully point out that the reference teaches aryl as naphthyl or preferably phenyl. There is no suggestion or motivation found in the reference for ring 1 to be anything else, such as pyrrole, pyrazol, pyridine, imidazole, pyrazoline or pyrimidine. As the court found in *Cardiac Pacemakers, Inc.* v. St. Jude Medical, Inc., 2004 U.S. App. LEXIS 18386, "Whether the prior art

provides the suggestion or motivation or teaching to select from prior knowledge and combine it in a way that would produce the invention at issue is a question of fact. *Winner Int'l Royalty Corp. v. Wang, 202 F.3d 1340, 1348 (Fed. Cir. 2000).*" Altmann et al. does not teach or suggest anything other than substituted or unsubstituted alkyl, cyclo-lower hydrocarbyl, cyclo-lower hydrocarbyl-lower alkyl, phenyl or naphthyl at the R_1 position. Atlmann et al. does not teach or suggest Applicants' genus.

The Examiner states:

> The closest prior art compound is that of Example 72 on page 35. This prior art compound differs from the sole compound Applicant's have exemplified by having a 4-Ph-OH substituent over the 4-Ph-OPh group of the instant compound at R_1. Applicants need to show that their compounds have superior and/or unexpected properties over the prior art compounds.

Applicants submit that the Examiner has not established a *prima facie* case of obviousness. The Examiner has not focused on the invention as a whole. Applicants' genus always contains a second cyclic moiety attached via a linker to the cyclic moiety attached at the nitrogen of the pyrrole ring. The compound cited by the Examiner contains no such second cyclic moiety.

Based upon the foregoing, the rejection of claims 1-40 and 44-88 under 35 U.S.C. §103(a) over Altmann et al. (WO 97/49706) is obviated and should be withdrawn.

Based upon the foregoing, Applicants believe that claims 1-35, 37-40, 44-45, 47 and 51-88 are in condition for allowance. Prompt and favorable action is earnestly solicited.

If the Examiner believes that a telephone conference would advance the condition of the instant application for allowance, Applicants invite the Examiner to call Applicants' agent at the number noted below.

Respectfully submitted,

Date: December 23, 2004

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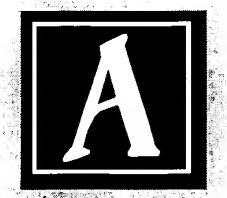
Exhibit A

Expert Opinion on Investigational Drugs



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Review

Tyrosine kinases in disease: overview of kinase inhibitors as therapeutic agents and current drugs in clinical trials

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Tyrosine kinases, first described as oncogenes, have been shown to play a role in normal cellular processes. Aberrations in tyrosine kinase activity lead to disease states. For fifteen years it has been postulated that the inhibition of tyrosine kinases may have therapeutic utility and the design and testing of inhibitors have been major focuses of research and development in both academic institutions and pharmaceutical companies. While early research focused on developing chemical entities that mimic phosphotyrosine, later research has focused on developing competitive adenosine triphosphate (ATP) inhibitors with various levels of selectivity on kinase targets. This review focuses on a discussion of tyrosine kinases thought to be important in disease, including platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial cell growth factor (VEGF), epidermal growth factor (EGF) receptors, HER-2 and Src. In addition, the classes of inhibitors designed to affect these targets and that have overcome research and development challenges and entered clinical trials are discussed. These include isoxazole, quinazoline, substituted pyrimidines and indolinone compounds, all of which are in clinical trials or near clinical development by SUGEN, Zeneca, Novartis, Pfizer and Parke-Davis. A summary of the chemistry and activity of these agents is provided.

Keywords: *angiogenesis, cancer, fibrosis, growth factors, indolinones, isoxazoles, psoriasis, pyrimidines, quinazolines, restenosis, tyrosine kinases*

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1. Introduction

Tyrosine kinases are enzymes that regulate signal transduction in cells, leading to mitogenesis, differentiation, migration, apoptosis and many other cellular functions. They exist as two major structural types, transmembrane receptors and cytoplasmic proteins. The receptor tyrosine kinases typically have an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase domain. Upon ligand binding, they dimerise and undergo a conformational change that activates the kinase, leading to transphosphorylation. Other substrates, adapter proteins and even cytosolic tyrosine kinases bind to the phosphorylated tyrosines of the receptor leading to a cascade of events that results in the final cellular function. Cytosolic tyrosine kinases may be activated by phosphorylation, as in the case of AKT, or by dephosphorylation, as occurs with Src family members.

As tyrosine kinases are involved in so many cellular functions, aberrant activity can lead to disease states. Such aberrant activity may be the result of

overactivation, as well as underutilisation of the signalling pathways. Many oncogenes found in cancers are derived from tyrosine kinase genes that have been deregulated, leading to constitutive activity [1,2]. Overexpression or activation of wild-type tyrosine kinases can also lead to cancer. Abnormal activation may result from an autocrine loop in which a growth factor and its receptor are expressed in the same cell, leading to continuous signalling [3]. Tyrosine kinases are also involved in restenosis, psoriasis, angiogenic diseases, immunological disorders and many other disease states, although their role in causative function is not always understood.

Type II diabetes is an example where too little signalling through a tyrosine kinase-mediated pathway leads to disease. In this case, lack of insulin receptor signalling results in a deficiency of cellular glucose uptake, causing hyperglycaemia. Other examples where a deficiency in signalling leads to pathological conditions include neuropathies, anaemias and immune suppression. This review will focus only on the overactivation of tyrosine kinases in disease and the development of tyrosine kinase inhibitors as therapeutic agents, which has led to a fast growing and competitive area of research (reviewed in [4]). Many tyrosine kinase inhibitors with different structural types have been introduced and these have recently been reviewed [5-9]. Here we discuss the major tyrosine kinases that have been validated as playing a role in disease and the advances that have been made in developing agents that inhibit them, with emphasis on the kinase inhibitors that have entered clinical trials.

2. Tyrosine kinases in disease states

2.1 Platelet-derived growth factor receptor

Platelet-derived growth factor (PDGF) is a major mitogen and chemoattractant for fibroblasts, smooth muscle cells and glial cells (reviewed in [10]). It exists as disulfide-linked homo- and heterodimers of A-chain and B-chain, resulting in three isoforms. These isoforms have differential binding affinities for the receptors, which are made up of α- and β-subunits. This family of receptors is characterised by Ig-like loops in the extracellular domain and a split kinase domain (reviewed in [11]). The receptors exist as homo- and heterodimers depending on which PDGF isoform is present. Signalling through both receptors causes phosphatidylinositol turnover, calcium flux and membrane ruffling, leading to cellular migration and proliferation.

The specific role of each receptor type is not fully understood, but recent studies of targeted mutagenesis in mice prove that, at least in development, there are distinct functions for the two receptor types. For example, mice embryos deficient in the α-receptor or the β-receptor do not survive until birth and embryos of both show evidence of bleeding, but each also causes specific defects [12,13]. Deficiency of the α-receptor causes severe bone abnormalities [13], whereas deficiency of the β-receptor results in kidney defects in embryos [12]. Furthermore, the β-receptor appears to play a role in angiogenesis by regulating the pericytes that surround vessels [14]. PDGF-AA and the α-receptor are required for gliogenesis during development [15]. PDGF is secreted from type-1 astrocytes [15] and neurones [16] and induces proliferation and differentiation of O2-A progenitors. In adults, PDGF plays a role in wound healing. Its chemotactic activity attracts fibroblasts, smooth muscle cells, monocytes and neutrophils to the site of injury (reviewed in [17]).

PDGF and its receptors have been implicated in a number of disease states including cancer. The PDGF B-chain gene was the first proto-oncogene to be identified when it was found to be the cellular homologue of v-*sis*, the transforming gene of simian sarcoma virus [18,19]. Subsequently, PDGF has been detected in many tumour cell lines, including breast [20] and colon cancers [21], and melanoma [22]. PDGF and its receptor are co-expressed in numerous tumour cell lines, such as sarcomas [23] and gliomas [22,24,25]. More importantly, the presence of PDGF and its receptors in tumour biopsies has been shown. In ovarian tumours, PDGF and the α-receptor were detected in malignant tumours by immunohistochemical staining, but they were not present in benign tumours or normal tissue [26]. Biopsies from human gliomas have been analysed by *in situ* hybridisation [27-29]. In high grade glioma, PDGF A-chain and PDGF α-receptors were co-expressed in tumour cells; they possibly function through an autocrine loop in such tumours. The β-receptor was expressed on endothelial cells of the tumours and B-chain was detected in both tumour and endothelial cells; paracrine and autocrine stimulation of receptors may occur in this situation.

The inhibition of PDGF signalling in tumour models has also been used to study the role of PDGF receptors in cancer. In a rat glioma cell line, introduction of a truncated PDGF β-receptor inhibited both *in vitro* growth and sc. tumour growth in athymic mice through a dominant-negative mechanism [30]. Similar results were found with mutant forms of PDGF A-chain [31,32]. These findings, along with the expression patterns, support the theory that PDGF and its receptors play a role in many types of human cancers.

PDGF has also been implicated in atherosclerosis [33] and restenosis [34]. *In vitro*, it is a chemoattractant and mitogen for smooth muscle cells, which are deposited in vessels in both conditions. In 30 - 40% of patients undergoing angioplasty to remove atherosclerotic plaques, restenosis occurs within six months. Balloon injury to the carotid artery of rats is utilised to mimic the process of restenosis, although this model has shortcomings. PDGF A-chain was quickly up-regulated in smooth muscle cells following injury and PDGF β-receptor mRNA increased gradually during the chronic phase of neointimal formation [35]. Furthermore, accumulation of smooth muscle cells in this model can be blocked by an antibody against PDGF [36]. It appears that PDGF stimulates the migration of smooth muscle cells, but not their proliferation. Restenosis is a complex process involving many factors, but PDGF plays a role in at least one aspect of it.

2.2 Fibroblast growth factor receptor

Fibroblast growth factor (FGF) receptors are related to PDGF receptors in that they have extracellular Ig-domains and a split kinase domain (reviewed in [37]). The receptors are coded for by four different genes and types 1 and 2 also have multiple isoforms due to alternative splicing. At least ten different forms of FGF have been identified [38-40]. All forms bind to heparin and heparan sulfate on cell surfaces. This association increases the affinity of FGF for its receptors. FGFs also bind to the extracellular matrix, which may serve as a storage site. FGF-1 and -2, also known as acidic and basic FGF, are the best characterised. Neither contains a signal sequence and the mechanism of secretion is not clear. Both bind to all four receptor types and stimulate the growth and migration of a number of cell types, including fibroblasts and endothelial cells.

As with PDGF, FGF plays a role in embryonic development. FGF-3 (INT-2) and FGF-4 (K-FGF/HST-1) are only expressed in embryos at specific time points [38]. FGF-6, which only binds to the type-4 receptor and FGF-2, appears to be involved in muscle development [38,41]. The expression pattern of FGF receptor-1 in mouse embryos is consistent with involvement in mesodermal patterning. Targeted mutagenesis of its gene caused a lethal phenotype in homozygotes with the embryos showing aberrant mesodermal patterning [42]. To investigate the role in skin development of the FGF receptor-2, which is the receptor for FGF-7 (keratinocyte growth factor), transgenic mice were developed that express a truncated form of the receptor in their epidermis [43]. The mutant receptor inhibited the wild-type receptor and caused abnormalities that suggest this receptor is required for normal keratinocyte differentiation. FGF receptor-3 is apparently involved in skeletal growth. Mutations in its gene have been identified in two types of dwarfism, achondroplasia [44,45] and thanatophoric dysplasia [46].

In the adult, the FGF receptor is involved in wound healing. FGF-2 increases the formation of granulation tissue and induces migration of fibroblasts and endothelial cells to the wound site [47]. FGF-7 is up-regulated by over 100-fold within 24 h of wounding and stimulates growth of keratinocytes [48]. Transgenic mice expressing an inhibitory form of FGF receptor-2 (the receptor for FGF-7) in their epidermis had delays in wound healing [49]. FGF also participates in wound healing by stimulating angiogenesis. FGF-1 and FGF-2 induce the release of proteases, and migration and proliferation of endothelial cells, all of which are required for angiogenesis (reviewed in [50,51]). FGFs have been shown to induce angiogenesis in a number of *in vivo* systems, including the chorioallantoic membrane of chicken embryos, corneas of mice and implants in rodents.

Just as FGF has similar biological functions to PDGF, it is also involved in the same diseases. FGF-1 and -2 and their receptors have been identified in a variety of tumour types. A human renal cell carcinoma cell line [52] and two human prostate tumour cell lines [53] produce FGF-2. In a panel of human oesophageal cancer cell lines, FGF-2 and FGF receptor-1 mRNAs were co-expressed; possibly an autocrine loop drives their growth [54]. In the skin of melanoma patients, high FGFR-1 expression was seen in invading melanoma cells and stroma, but not in the endothelial cells [55]. Analysis of astrocytomas showed that mRNA expression for type 1 and type 2 FGF receptors changes as tumours progress to higher grades [56].

FGFs and their receptors are expressed in tumour endothelium, as well as tumour cells. They are likely to play a role in both the proliferation of tumour cells and tumour angiogenesis.

Neutralising antibodies against FGF-2 have been used to confirm that it has a functional role in cancer. An antiFGF-2 antibody blocked mitogenesis of SC115 mouse mammary carcinoma cells in response to FGF-1 [57]. Similarly, a monoclonal FGF-2 antibody inhibited the growth of U-87MG and T98G human glioblastoma cells in culture and in nude mice [58].

FGF also stimulates vascular smooth muscle cells to migrate and proliferate in restenosis [34]. FGF-1 and FGF-2 are released from dying smooth muscle cells during angioplasty. Furthermore, mRNA for FGF-2 and FGF receptor-1 are up-regulated in smooth muscle cells after injury and may act by an autocrine mechanism [59]. A neutralising antibody against FGF-2 inhibited early smooth muscle cell proliferation in a balloon injury model, although it did not reduce the size of the intimal lesion [60]. As with PDGF, FGF is important for restenosis, but it does not act alone.

2.3 Flk-1/KDR

Flk-1 is structurally related to PDGF and FGF receptors with seven immunoglobulin-like sequences in the extracellular domain and a split tyrosine kinase domain. It is a receptor for vascular endothelial cell growth factor (VEGF) [61,62]; KDR is its human homologue [63]. Other members of the family are Flt-1 [64,65], which also binds VEGF, and Flt-4 [66]. Flk-1/KDR and Flt-1 are expressed primarily on vascular endothelium and Flt-4 is expressed on lymphatic endothelium. VEGF stimulates growth of endothelial cells during the process of angiogenesis, the sprouting of new blood vessels from pre-existing vessels (reviewed in [67]). It was also independently isolated as vascular permeability factor (VPF) (reviewed in [68]). VEGF has four different splice variants and exists as a disulfide-linked homodimer with structural similarities to the PDGFs. Recently, two new members of the VEGF family have been identified, VEGF-B and VEGF-C. VEGF-B stimulates the growth of endothelial cells, but its receptor, or receptors, is not yet known [69]. VEGF-C, also known as vascular endothelial growth factor-related protein, was identified as a ligand for Flt-4 [70,71], but it also binds to Flk-1/KDR. There is some evidence that co-expression of two VEGF family members in the same cell type leads to the formation of heterodimers. The functions of such proteins remain to be elucidated.

VEGF and its receptors are clearly involved in angiogenesis during development. Binding of [^{125}I]-labelled VEGF [72] and *in situ* hybridisation have been used to show that VEGF [73] and its receptors [62] are expressed in vascular endothelium in mouse embryos. Furthermore, targeted deletion of the Flk-1 gene resulted in embryos with a defect in haematopoietic and endothelial cell development that led to a lack of organised blood vessels [74]. In normal adults, angiogenesis only occurs in wound healing, corpus luteum formation and pregnancy. Correspondingly, Flk-1 was not found to be expressed in vessels of normal adult mice [62].

Angiogenesis is required for tumours to grow beyond a minimum volume and to switch to a neoplastic phenotype [75]. The roles of VEGF and Flk-1/KDR are well defined in this process. VEGF is secreted by a number of human tumour cell lines in culture, such as glioma [76], melanoma [77] and epidermoid carcinoma cells [78]. More importantly, VEGF transcripts or protein have been identified by *in situ* hybridisation or immunohistochemistry in primary gliomas [79,80], haemangioblastomas [81], and breast [82-84], colon [85,86] and renal cell tumours [87]. Like VEGF, mRNA for Flk-1/KDR has been detected in tumours, such as gliomas [79,80], haemangioblastomas [81], colon cancer [86] and adenocarcinomas [85]. In all cases, the receptors were detected on the endothelial cells of the vessels and not the tumour cells. This supports a paracrine mechanism in which VEGF secreted from tumour cells stimulates proliferation of endothelial cells.

A number of animal models have been developed to investigate the function of VEGF and Flk-1/KDR in tumour angiogenesis. The introduction of antisense constructs against VEGF into rat C6 glioma [88] and human U87MG glioblastoma cells lines [89] reduced their sc. growth in athymic mice, as well as the degree of neovascularisation. Monoclonal antibodies against VEGF have also been utilised to inhibit the sc. growth of several human tumour cell lines in athymic mice [90,91]. Truncated Flk-1 was used to study the capacity of Flk-1 to act as a modulator of tumour growth in animal models. Athymic mice were co-implanted with tumour cells and virus-producing cells that carry the mutant *flk-1* gene [92,93]. This allowed the introduction of mutant receptor into endothelial cells, where it acted by a dominant-negative mechanism to block

activation of Flk-1. By this method, the sc. growth of a variety of human, rat and mouse tumour cells was inhibited. The vessel density was also reduced in the small tumours that did form, which confirmed the connection between Flk-1, angiogenesis and tumour growth.

Angiogenesis occurs in other disease states, such as haemangioblastoma formation, psoriasis and diabetic retinopathy. VEGF and Flk-1 have been shown to play a role in these situations, as well as in cancer [67]. In two studies, haemangioblastomas, which are highly vascularised tumours, were shown to express VEGF, Flk-1/KDR and Flt-1 [81,94]. All three proteins were also found to be highly expressed in psoriatic skin, but not in normal skin [95]. Psoriatic lesions are hyperpermeable as well as highly vascularised. VEGF is up-regulated in the ocular fluid of patients with diabetic retinopathy [96]. It is apparently induced by hypoxia, which occurs as a result of vessel damage in the retina. VEGF signalling through Flk-1/KDR is involved in a number of angiogenic diseases, but occurs only rarely in normal adults. This makes Flk-1/KDR an ideal target for therapeutic intervention.

2.4 Epidermal growth factor receptor

The epidermal growth factor (EGF) receptor was cloned in 1984 by Ullrich *et al.* [97]. It has two cysteine-rich regions in the extracellular domain and a single kinase domain. There are three other known members of the EGF receptor family, HER-2, HER-3 and HER-4 (also known as erbB2, erbB3 and erbB4). Besides EGF, several other ligands bind to the EGF receptor: transforming growth factor-α (TGF-α), betacellulin, amphiregulin, heparin-binding EGF (HB-EGF) and neuregulin (also known as heregulin). EGF and betacellulin induce formation of EGF receptor heterodimers with the other family members, as well as EGF receptor homodimers. Likewise, betacellulin is a ligand for HER-4 as well as the EGF receptor and binds to heterodimers containing either receptor.

Unlike PDGF and FGF receptors, the EGF receptor is not absolutely required for development. Although deletion of the EGF receptor gene can be lethal in some genetic backgrounds, some strains of mice have survived up to three weeks after birth despite a deficiency in the EGF receptor [98,99]. The mice were born with eyes opened, rudimentary whiskers and epidermal defects. They also had abnormalities in kidney, brain, liver and the gastrointestinal tract; thus the EGF receptor is involved in the development of several organs.

Members of the EGF receptor family and its ligands are overexpressed, or expressed as an autocrine loop in many tumour types. Amphiregulin was found to be co-expressed with the EGF receptor in pancreatic cancer cells [100] and ovarian carcinoma specimens [101]. Ten out of 13 renal cell lines studied express TGF-α along with the EGF receptor [102]. HB-EGF was expressed in an autocrine loop in gastric cancers and cell lines [103] and hepatocellular carcinomas [104]. In a study of primary breast tumours, 59% of the samples expressed the EGF receptor and its expression correlated with nonresponsiveness to hormone therapy [105]. In most cases, overexpression of the EGF receptor does not result from gene amplification, but the gene was amplified in some glioblastomas [106].

Not only is the EGF receptor present in many tumours, it may be required for tumour cell growth. This was shown with an inhibitory antibody against the receptor, which blocked the growth of tumour cell lines, both *in vitro* and as xenografts in athymic mice [107,108]. Furthermore, an antisense oligonucleotide for amphiregulin inhibited the growth of a pancreatic cancer cell line [100]. The frequent occurrence of the EGF receptor in an autocrine loop in cancer makes it a good target for chemotherapy.

Several lines of evidence suggest a role for the EGF receptor system in psoriasis, a disease characterised by disregulation of keratinocyte cell growth with epidermal hyperplasia. The TGF-α/EGF receptor system is an excellent example of autocrine activation leading to epidermal keratinocyte proliferation. TGF-α is produced by keratinocytes in normal human epidermis and cultured keratinocytes [109,110]. However, it is overexpressed in psoriatic epidermis as determined by immunohistochemistry and mRNA assays [109-115]. Studies using [^{125}I]-EGF have shown that the normal basilar distribution of EGF receptors in epidermal keratinocytes was markedly altered in *psoriasis vulgaris* where they were also observed in the upper keratinocyte compartment [115,116]. In a study on benign epidermal dermatoses, EGF receptor expression throughout the epidermis returned to a basal layer distribution when the lesion resolved [117]. Finally, it has been shown that other cytokines and growth factors, such as IFN-γ, TNF-α, IL-8 and IGF-1, induced expression of TGF-α, EGF receptor or both [118,119]. These data suggest that inhibition of EGF

receptor signalling may provide therapeutic benefit in psoriasis by controlling the hyperproliferation of psoriatic keratinocytes.

2.5 HER-2

The rat homologue of HER-2, Neu, was originally identified as a transforming protein in ethylnitrosourea-treated rats [120]. The oncogene has a point mutation in the transmembrane domain that codes for a mutant receptor-like protein that dimerises constitutively [121], resulting in activation of the tyrosine kinase. The human homologue, HER-2, was found in an effort to clone EGF receptor-related genes [122,123]. It remains an orphan receptor despite years of effort to identify a ligand. It is constitutively activated when overexpressed even without the activating mutation found in Neu [124,125]. It forms heterodimers with the EGF receptor in the presence of EGF [126] and with HER-3 and HER-4 in the presence of neuregulins [127,128]. HER-2 is activated when heterodimerised with other family members and is the preferred partner for the other receptors [129].

The normal role of HER-2 is not clear. It may regulate the activities of EGF and the neuregulins by interacting with their receptors. Potentially, it is involved in differentiation and mitogenesis of many cell types. Its role in cancer is much better understood. It has been found to be overexpressed in about 30% of breast and ovarian cancers and expression has been correlated with poor prognosis (reviewed in [130]). In some cases, overexpression is a result of gene amplification. Recently, the HER-2 gene was also found to be amplified in many prostate carcinomas and this correlated with tumour grade and disease recurrence [131]. Higher expression was also seen in colorectal adenocarcinoma than in benign lesions [132]. As well as contributing to proliferation of tumour cells, HER-2 may protect them from the immune system by inducing resistance to TNF-α [133] and lymphokine-activated killer cells [134].

Extensive research has been done with inhibitory antibodies against HER-2. In NIH 3T3 cells transformed by Neu, a monoclonal antibody down-regulated Neu and reverted the transformed phenotype [135]. In AU-565 human breast cancer cells, an antiHER-2 antibody induced differentiation [136]. Antibodies were also effective at inhibiting the growth of sc. tumours resulting from implantation of HER-2-expressing NIH 3T3 cells in athymic mice [136,137]. These results confirm that HER-2 plays a role in cancer and that it is an excellent target for intervention. Indeed, a humanised monoclonal antibody against HER-2 is currently in clinical trials by Genentech. In a small Phase II trial, 11.6% of patients responded and 37.2% had stable disease after ten doses [138]. Phase III studies are expected to be completed this year.

2.6 Src Family

The first oncogene to be discovered was v-*src*, the transforming gene of the Rous sarcoma retrovirus. Its cellular homologue, c-*src*, was later found and both were determined to code for tyrosine kinases. There are now nine known members of the Src family: Src, Fyn, Yes, Lck, Lyn, Fgr, Hck, Blk and Yrk. Unlike the receptor tyrosine kinases, the Src family of tyrosine kinases lack ligand binding and transmembrane domains. They contain Src homology 2 and 3 domains, known as SH2 and SH3 domains, which mediate interactions within the protein and with other proteins. These domains are also found in many other proteins and their specific binding sequences are well known [139]. SH2 domains bind to phosphotyrosine residues with flanking amino acids that are specific for the particular SH2 sequence. SH3 domains bind to proline-rich regions. Each Src family member also has a unique domain near the amino terminus and is associated with the membrane by myristilation.

Src family members are regulated by phosphorylation of a tyrosine in the carboxy-terminal tail. Dephosphorylation of this tyrosine allows a conformational change that leads to kinase activation. Recently, the crystal structure of inactive Src was analysed to reveal in more detail how Src is regulated [140]. Not only does the SH2 domain associate with the phosphotyrosine at 527, but the SH3 domain locks the protein in the inactive state by associating with the linker between the SH2 and kinase domains. Although Src family members are not activated directly by ligands, they are activated in response to PDGF, EGF and other growth factors. In the case of the PDGF receptor, Src family members bind through their SH2 domains to two tyrosine residues in the juxtamembrane domain of the PDGF receptor [141-143]. Src is activated and, in turn, phosphorylates tyrosine 934 of the PDGF β-receptor [144]. PDGF receptors mutated at this site have decreased mitogenic responses to PDGF-BB. Furthermore, introduction of a kinase inactive Fyn construct into NIH 3T3 cells inhibits PDGF-induced mitogenesis by blocking the association of active Src family members to the PDGF receptor [145].

Figure 1: Structure of SU101 and its major metabolite SU0020.

SU101
Leflunomide
HWA 486

SU0020
A771726
RS-61980-00

Src is also involved in signalling by EGF receptor family members. Co-expression of the EGF receptor and Src in murine fibroblasts was used to show that Src associates with the EGF receptor and that EGF-induced mitogenesis was increased in the presence of Src [146]. Furthermore, the co-expressing cells were able to grow in soft agar and athymic mice. In another system with activated Neu expressed under an inducible promoter, it was shown that Src binds to phosphorylated Neu increasing its kinase activity [147]. Direct interaction between Src and the EGF receptor was not detected; they may associate through Neu.

Despite the fact that Src is involved in mitogenic signalling from growth factor receptors, deletion from mouse embryos is not lethal [148]. This may be because Fyn, Yes or other family members can substitute for Src. The mutant mice died within a few weeks of birth and were found to have impaired osteoclast function, indicating that Src may play a role in bone remodelling.

Src family members are also thought to be involved in cancer, particularly of the colon. In colon carcinoma cell lines and biopsy samples, Src was found to be highly activated compared to normal colonic mucosal cells and normal tissue [149]. Yes was also found to be elevated in another set of samples, while other family members, Lck, Fyn, Hck, Lyn and Fgr, were not [150]. It has also been shown that Yes kinase activity was higher in colonic adenomas that had signs indicating that they were at risk for malignancy [151]. In a recent study, antisense against Src was able to not only reduce the level of Src in the human colon tumour cell line HT 29, but also to reduce their proliferation rate *in vitro* and tumourigenesis in athymic mice [152]. Although both Src and Yes appear to play a role in colon cancer, only Yes was found to have elevated activity in a panel of melanoma cell lines compared to normal melanocytes [153]. Src family members may be able to substitute for each other in normal cells, but, in cancerous cells, only one or two members may contribute to the transformed phenotype.

3. Tyrosine kinase inhibitors

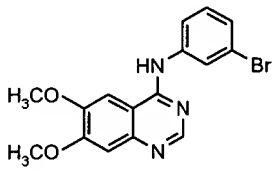
Several review articles have recently been published that describe the different classes of tyrosine kinase inhibitors [5-9]. In this section, the inhibitors that have overcome the preclinical challenges and entered clinical development will be discussed. Therefore, while kinase inhibitors, such as quinolines, quinoxalines, tyrphostins, biarylhydrazones, and natural products, such as erbstatin, genistein, herbimycin A and geldanamycin, have been important historically and served as the basis for much of the early development of kinase inhibitors, they will not be reviewed here.

3.1 SU101/leflunomide

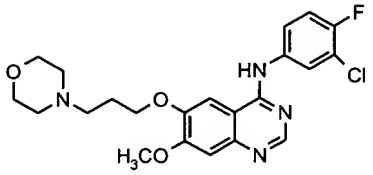
N-[(4-trifluoromethyl)-phenyl]-5-methylisoxazole4--carboxamide is in clinical trials by SUGEN (SU101) for cancer and Hoechst AG (Leflunomide) for rheumatoid arthritis. SUGEN has begun a Phase III trial in recurrent malignant glioma and Hoechst AG has completed or is near completion of its Phase III trial in rheumatoid arthritis. The compound has been studied by several laboratories, where activity has been ascribed to two areas, inhibition of tyrosine kinases and inhibition of pyrimidine biosynthesis. The isoxazole compound is rapidly converted *in vivo* to the metabolite, 2-cyano-3-hydroxy-N-(4-trifluoromethylphenyl)butenamide, which is known by several names as shown in **Figure 1**.

The literature describes activities associated with both the closed-ring parent compound and the open-ring metabolite, although most studies have focused on the metabolite. The open-ring structure has been reported to have effects on a number of tyrosine

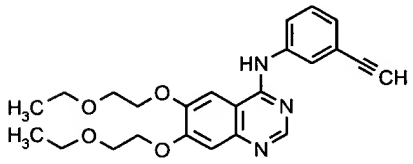
Figure 2: Quinazolines in Phase I clinical trials.



PD 153035/SU5271



ZD1839



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kinases, including Fyn and Lck, and tyrosine phosphorylation of the T-cell receptor ζ chain induced by antiCD3 monoclonal antibodies [154-156]. In addition, Mattar *et al.* found that phosphorylation of the EGF receptor was inhibited by the open-ring metabolite [157]. Elder *et al.* found inhibition of IL-2-induced tyrosine phosphorylation of JAK1 and JAK3, as well as tyrosine phosphorylation of the β-chain of the IL-2 receptor [158]. Shawver *et al.* showed that PDGF-mediated signalling was inhibited by SU101, including receptor phosphorylation, cell cycle, DNA synthesis, and tumour cell growth [159]. The latter studies focused on the parent, closed-ringed structure rather than the open-ring metabolite.

In addition to inhibiting tyrosine kinases, the open-ring metabolite has been shown to inhibit pyrimidine biosynthesis *via* inhibition of dihydroorotate dehydrogenase, the fourth enzyme of *de novo* pyrimidine biosynthesis [160-162]. The effects on pyrimidine biosynthesis can be overcome by the addition of exogenous uridine to cell cultures, to replenish the nucleotide pools [158,160,161]. However, the addition of uridine does not overcome the effects on proliferation due to antagonising tyrosine kinase activity [156,158,159]. Thus, the anti-inflammatory and anticancer properties of this compound are likely due to at least two separate and distinct activities. It has recently been shown [Lipsom *et al.*, manuscript in preparation] that the PDGF-mediated activity of SU101/leflunomide can be ascribed to the parent molecule, and the route of drug administration influences the ability to deliver its anticancer activity *in vivo*. Whereas broad activity was observed following parenteral administration, inhibition of PDGF-mediated tumour growth was not observed following oral administration, most likely due to conversion to the metabolite in the gastrointestinal tract. Thus, it appears that only parenteral administration of the compound retains its ability to inhibit PDGF-mediated cancers.

3.2 Quinazolines

Quinazolines have been studied extensively in several pharmaceutical companies and three are currently in clinical development (**Figure 2**). PD 153035 was published in 1994 [163], as a compound that inhibits the EGF receptor phosphorylation at picomolar concentrations and HER-2 at low micromolar concentrations, but only had effects on the PDGF and FGF receptors at 50 μM. Hence, this compound is a very selective tyrosine kinase inhibitor. This is in contrast to the structurally similar quinolines and quinoxalines, early tyrosine kinase inhibitors, which differ in the position and/or number of the nitrogens in the rings and show poor selectivity in comparison. Other structure-activity studies examined the distance between the amine and the phenyl group, and substituents on both the quinazoline and aniline moieties [164-166]. As with quinolines and quinoxalines, 6,7-dimethoxy-substituted quinazoline compounds were the most potent, although dihydroxy- and diamino-substituted quinazolines were also active [164,165]. Halogens in the meta position of the aniline group were the most effective at inhibiting the EGF receptor in kinase assays [165,166]. The combination of electron donating groups in the 6- and 7-positions and a halogenated aniline group has a 'supra-additive' effect, in which the potency was much greater than expected [165]. Growth of KB tumour cells was inhibited by quinazolines, and 4-(3-chloroanilino)quinazoline inhibited EGF-stimulated, but not PDGF- or IGF-stimulated, growth of other cell lines [166]. Not only is PD 153035 active in *in vitro* EGF receptor tyrosine kinase assays, but a single dose reduced the

phosphorylation of the EGF receptor in tumours derived from implantation of A431 cells in athymic mice [167]. Parke-Davis is not pursuing clinical trials of PD 153035 in cancer, but the compound is also known as SU5271 and is in Phase I clinical trials by SUGEN as a topical agent for the treatment of psoriasis. As discussed previously, the EGF receptor is thought to be a primary driver of keratinocyte proliferation through activation by either EGF or TGF-α. Since this compound is one of the most potent EGF receptor inhibitors known, it may provide proof-of-concept for the role of EGF in psoriasis.

ZD 1839 is under development by Zeneca for the treatment of cancer and is in Phase II studies. Like other quinazolines, it is a potent EGF receptor inhibitor, exhibiting an IC_{50} of 23 nM for tyrosine kinase activity and 80 nM for inhibition of KB cell proliferation [168]. It was shown to be a competitive ATP inhibitor with a K_i = 2.1 nM on purified EGF receptors. ZD 1839 showed excellent activity against xenografts in athymic mice. When administered orally at 10 mg/kg/day, a 50% reduction in the growth of A431 cells was observed. Activity was also shown against A549 (lung), Lovo (colon) and DU145 (prostate) tumours. Perhaps surprising, however, was the observation that 200 mg/kg/day caused regression of 1.5 g A431 tumours to undetectable sizes within two weeks, and tumour growth was suppressed for as long as 4 months. Tumour regression would not be expected for a cytostatic growth factor receptor inhibitor. However, regrowth occurred when treatment was suspended.

CP-358,774 is a quinazoline under clinical development by Pfizer [169,170]. It is also a potent EGF receptor tyrosine kinase inhibitor (IC_{50} = 2 nM) and shows greater than 1000-fold selectivity against other tyrosine kinases such as $pp60^{v\text{-}src}$, $pp145^{c\text{-}abl}$, the insulin receptor and the IGF-1 receptor. It has been shown to be a reversible ATP competitive inhibitor. Daily oral administration at 10 mg/kg to athymic mice bearing HN5 (head and neck tumour cells) xenografts resulted in 50% inhibition. For inhibition of growth of A431 derived tumours, a higher dose of CP-358,774 was needed (200 mg/kg/day). As well as inhibiting tumour cell growth, CP-358,774 induced apoptosis in DiFi human colon carcinoma cells. It is not yet known whether this also occurs in *in vivo* tumour models [171].

The quinazolines represent an important class of tyrosine kinase inhibitors, particularly with regard to the EGF receptor family. Because heterodimer formation between the EGF receptor and HER-2 leads to mitogenesis [129], inhibiting both tyrosine kinases may be more effective than blocking only the EGF receptor. PD 153035 inhibited both receptors [163], but HER-2 activity was not reported for ZD1839 or CP-358,774. At least three quinazolines are currently in clinical trials and results will provide important data on the therapeutic potential of this class of drugs. An open issue at this juncture is the pharmacokinetic profile in man; many of the quinazolines exhibit poor pharmacokinetics in animal models, although medicinal chemistry approaches have attempted to improve these properties. It remains to be determined in clinical trials whether the improvements are sufficient, and to gain an understanding of the pharmacokinetic/pharmacodynamic relationship.

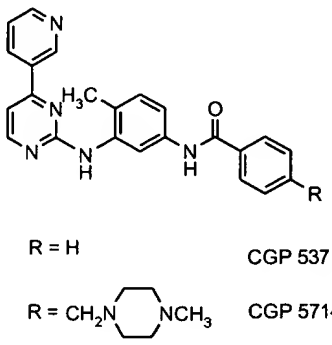
3.3 Substituted pyrimidines

Several substituted pyrimidines are in clinical development, or near clinical development. In addition to the quinazolines, they represent an important class of tyrosine kinase inhibitors. However, they may have broader application in their ability to inhibit, selectively or nonselectively, a greater number of targets. The substituted pyrimidines are comprised of pyrido-, pyrrolo-, pyrazolo-, pyrimido- and phenylaminopyrimidines. Novartis has a phenylaminopyrimidine in clinical development (CGP 57148) for the treatment of leukaemia and Parke-Davis has a number of pyridopyrimidines in late stage preclinical testing.

3.4 Phenylaminopyrimidines

Novartis synthesised a series of 2-phenylaminopyrimidines and pursued CGP 53716 as representative of the class [172]. This compound inhibited tyrosine phosphorylation of the PDGF receptor in *in vitro* and cellular assays with an IC_{50} of 0.1 μM in both types of assays. It was specific relative to the EGF receptor and a number of other tyrosine and serine/threonine kinases. The only other kinase it was found to potently inhibit was v-Abl. The cytoplasmic tyrosine kinase, v-Abl, was originally isolated as the product of the transforming gene of Abelson murine leukaemia virus [173]. Abl is also found as a fusion product with Bcr, resulting from a translocation between chromosomes 9 and 22 in human chronic myelogenous leukaemia [174]. CGP 53716 was orally efficacious at inhibiting the growth of v-*sis* and c-*sis* transformed BALB/c 3T3 cells in athymic mice, but did not inhibit

Figure 3: The structures of CGP 53716 and CGP 57148 (a phenylaminopyrimidine in Phase I clinical trials).



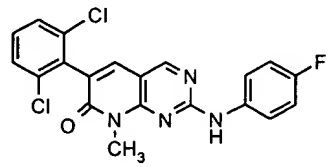
the growth of the EGF receptor-driven A431 cells as sc. tumours.

Novartis has pursued clinical studies with CGP 57148, a close analogue of CGP 53716 (**Figure 3**). This compound is also an inhibitor of the Abl and PDGF receptor kinases [175] with 30- to 100-fold selectivity when compared against the EGF, insulin growth factor-1 (IGF-1) and insulin receptor kinases. In intact cells, CGP 57148 inhibited v-Abl tyrosine kinase with an IC_{50} of 0.1 - 0.3 μM, with similar potency against the PDGF receptor. CGP 57148 exhibited antitumour activity *in vivo* in xenograft models. Doses of 3.13 - 50 mg/kg/day by ip. administration inhibited the growth of MuLV and v-*sis* transformed BALC/c 3T3 cells, with the highest dose almost achieving tumour stasis after 28 - 31 days of treatment.

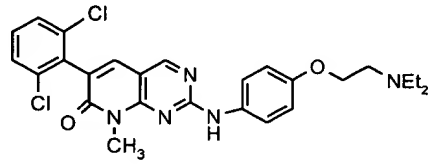
3.5 Pyridopyrimidines and pyrimidopyrimidines

Pyridopyrimidines, synthesised by Parke-Davis, are similar to quinazolines except that they have an additional nitrogen atom in the ring system. In the initial publication, a series of 7-aminopyrido[4,3-*d*]pyrimidines were compared for inhibitory activity on the EGF receptor [176]. As with the quinazolines, substitution in the meta position on the aniline ring increased potency compared to other positions, resulting in IC_{50} values of 0.01 - 1.5 μM. Methyl groups on the 7-amino group also resulted in nanomolar IC_{50} values [177]. Water solubility was increased while retaining inhibitory activity by the addition of a morpholine to the 7-amino group [178]. The position of the 'extra' nitrogen was also investigated [177]. Pyrido[3,4-*d*]pyrimidines were very similar to pyrido[4,3-*d*]pyrimidines at inhibiting the EGF receptor, but pyrido[2,3-*d*]- and pyrido[3,2-*d*]

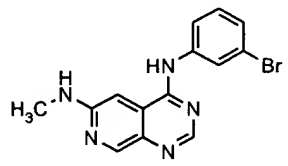
Figure 4: Representative pyridopyrimidines.



PD 173956



PD 166285



PD 158780

pyrimidines in this study were much less active, with the best IC_{50} being 3.1 μM. Subsequently, 6-phenyl substituted pyrido[2,3-*d*]pyrimidines were found to be potent inhibitors of the PDGF receptor, FGF receptor and Src [179]. Potency and specificity were altered by changing the substituents on the phenyl group.

Pyrimido[5,4-*d*]pyrimidines were synthesised to evaluate the effect of including an additional nitrogen atom in the ring system [180]. A series of analogues were prepared with electron-donating substituents in the 6-position and compared to quinazolines, pyrido[3,2-*d*]pyrimidines and pyrido[3,4-*d*]pyrimidines with the same substitutions. Although the pyrimido[5,4-*d*]pyrimidines were more potent in an *in vitro* EGF receptor kinase assay than pyrido[3,2-*d*]pyrimidines (1.5 *vs.* 7.6 nM for 7-amino-substituted), they followed the same pattern, i.e., there was no large increase in potency by adding methyl groups to the 7-amino, as was seen in the pyrido[3,4-*d*]pyrimidines. Crystal structure analysis indicated that the pyrido[3,4-*d*]pyrimidines had a conformational change to relieve the interactions between the hydrogen atoms on the carbon in the 5-position and the nitrogen in the 9-position. This

apparently allowed a 'supra-additive' effect with these compounds, such as was seen with quinazolines.

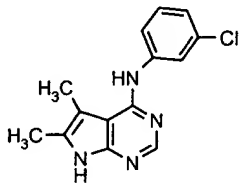
PD 158780 (**Figure 4**) is a pyridopyrimidine that inhibits purified EGF receptor tyrosine kinase with an IC_{50} = 8 pM and EGF receptor autophosphorylation in A431 cells at 13 nM [177,181]. Micromolar concentrations were required to inhibit PDGF- and bFGF-dependent processes in Swiss 3T3 cells, compared to low nanomolar for inhibition of EGF-dependent processes. PD 158780 was found to inhibit heregulin-stimulated phosphorylation in SKBR3 and MDA-MB-453 cells in the nanomolar range (IC_{50} = 49 and 52 nM). *In vivo* tumour growth was studied using A431 and MCF-7 xenografts, where 58% and 73% inhibition was achieved, respectively. A second pyridopyrimidine studied by Parke-Davis, PD173956, was found to inhibit c-Src activity with an IC_{50} of 26 nM, with selectivity compared to PDGF, FGF and EGF receptors (IC_{50} > 1 μM). *In vitro* antitumour cell activity was shown against colon cell lines with IC_{50} = 500 - 800 nM [181].

A third pyridopyrimidine, PD 166285, was shown to have impressive activity against PDGF, FGF and EGF receptors (IC_{50} = 39.3 - 98.3 nM) as well as Src family members, for which it was most potent. An IC_{50} = 8.4 nM on c-Src and IC_{50} < 1 nM on Fyn, Lyn and Lck was observed [183]. Broad *in vivo* activity was observed with PD 166285 against NIH 3T3/PDGF transfectants, C6 (rat glioma), HT29 (human colon) and SKOV-3 (human ovarian) cells. Recently, Hamby *et al.* [184] conducted SAR studies around the C-2 amino and C-6 aryl positions of the pyridopyrimidines and found compounds with improved potency, solubility and bioavailability, important considerations for clinical development candidates. 3',5'-Disubstituted phenyl compounds were selective inhibitors of FGF receptor tyrosine kinase with an IC_{50} of 0.77 μM compared to > 50 μM for PDGF and EGF receptors and c-Src.

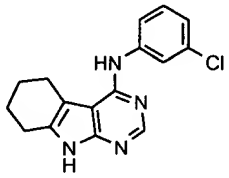
3.6 Pyrrolopyrimidines and pyrazolopyrimidines

Pyrrolo- and pyrazolopyrimidines have a five-membered ring fused to a six membered ring, instead of two six-membered rings. Novartis designed pyrrolopyrimidines to fit its pharmacophore model for the binding of dianilinophthalimides [185] into the ATP binding site of the EGF receptor [186]. In *in vitro* assays, CGP 59326, CGP 60261 and CGP 62706 (**Figure 5**) were potent for inhibition of the EGF receptor with IC_{50} values less than 30 nM and were specific relative to c-Src, v-Abl and protein kinase C.

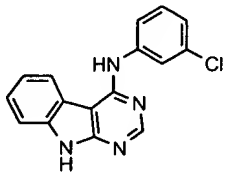
Figure 5: Representative pyrrolopyrimidines.



CGP 59326



CGP 60261

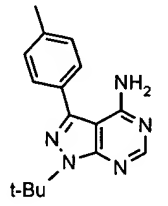


CGP 62706

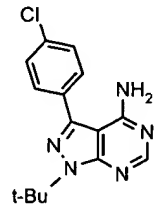
They also inhibited EGF receptor phosphorylation in EGF-stimulated BALB/c 3T3 cells, but not PDGF receptor phosphorylation with PDGF treatment. CGP 59326 was efficacious as a single agent and in combination with cytotoxic agents in several EGF receptor expressing-human xenograft models. This compound is close to entering clinical trials [5].

Pyrazolopyrimidines have one more nitrogen atom in the five-membered ring than pyrrolopyrimidines. Pfizer has used two such compounds to study the role of Src family members, Lck and Fyn, in T-cell activation [187]. PP1 and PP2 (**Figure 6**) inhibited Lck and Fyn in *in vitro* assays with IC_{50} values around 5 nM. They did not inhibit ZAP-70 or JAK2, and potency on the EGF receptor was about 120- to 200-fold less than on the Src family members. PP1 also inhibited antiCD3-stimulated tyrosine phosphorylation and proliferation of human T-cells. Novartis is optimising pyrazalopyrimidines as EGF receptor tyrosine kinase inhibitors [186].

Figure 6: Structures of pyrazolopyrimidines.



PP1



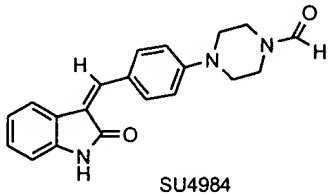
PP2

The substituted pyrimidines are beginning to enter clinical testing (e.g., CGP 57148) and it is likely that several additional molecules will enter clinical development soon. Unlike the quinazolines, which target only the EGF receptor family, substituted pyrimidines may have broader application. Activity has been observed against PDGF receptor and Src family members. In addition, they have the potential to hit a number of targets, as exemplified by the activity of PD 166285 against PDGF, FGF and EGF receptors and c-Src. If such a compound exhibited a good toxicological profile, it may have potential for broad applicability in treating cancer (see section 4). They may also be useful therapeutics for restenosis, where the PDGF and FGF receptors both play a role.

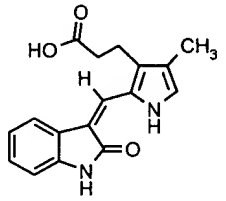
3.7 Indolin-2-ones

In addition to the substituted pyrimidines, the 3-substituted indolin-2-ones represent a second class of tyrosine kinase inhibitors that have the potential for broader target activity. Three 3-substituted indolin-2-ones have been described (**Figure 7**) by SUGEN, one of which is in clinical development. SU4984 and SU5402 were co-crystallised with the FGF receptor type 1 kinase domain [188]. Analysis of the structures showed that these compounds bind to the ATP binding site with the indolin-2-one ring system in the site for the ATP adenine. The side-chains on the indolin-2-one core conferred specificity to the FGF receptor tyrosine kinase. SU4984 inhibited the PDGF, EGF and insulin receptors, as well as the FGF receptor. In the crystal structure, hydrogen bonding

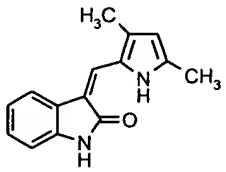
Figure 7: Structures of 3-substituted indolin-2-ones.



SU4984



SU5402



SU5416

between the indolin-2-one core and the enzyme was found only to peptide backbone atoms that are conserved in tyrosine kinases. SU5402, which specifically inhibits the FGF receptor, formed additional hydrogen bonds from the carboxylic acid group to Asp 568, which is specific for the FGF receptor.

SU5416 is currently in clinical development by SUGEN as an anti-angiogenic agent. It is a potent Flk-1 tyrosine kinase inhibitor [189; Fong *et al.*, manuscript in preparation] with an IC_{50} of approximately 1 μM in a cell-based assay. It has 20-fold selectivity against the PDGF receptor and 100-fold selectivity against the EGF receptor, HER-2, and IGF-1 receptor. Suprisingly, it does not inhibit FGF receptor kinase activity despite its similarity to SU5402. Apparently, substitutions on the pyrrole ring are important for conferring specificity. SU5416 inhibited the proliferation of endothelial cells stimulated with VEGF, with an IC_{50} of 40 nM. The compound has also shown broad-range activity in inhibiting xenografts in athymic mice at doses of ≤ 25 mg/kg/day. Additional indolin-2-one compounds with different target profiles are currently in preclinical development at SUGEN.

4. Future directions

With the discovery of oncogenes, particularly of the tyrosine kinase family, as components of signal transduction pathways came the speculation that inhibitors may provide therapeutic benefit to cancer patients. Since that time, it has become clear that tyrosine kinases play a role in diseases outside of cancer, which might benefit from drugs that target these enzymes. For example, it is clear that anti-angiogenic tyrosine kinase inhibitors will have broad applicability in diseases such as rheumatoid arthritis, ocular diseases of neovascularisation (macular degeneration, diabetic retinopathy), psoriasis and restenosis [190].

Over the past fifteen years, several issues have been raised that posed potential roadblocks to the development of such agents. Although most questions are being addressed in the context of treating cancer patients, the answers will certainly have implications for the other diseases where kinases play important roles. The first question that was raised was one of expression, comparing normal cells or tissue to their cancerous counterparts. It was reasoned that if expression could be found to be limited to the cancerous cells, the likelihood of success in developing a tyrosine kinase inhibitor might be greater. Many studies have examined the expression of tyrosine kinases in normal *versus* cancerous tissue. Indeed, some were found to be overexpressed compared to normal cells, but few have been found to be solely expressed in cancer cells. Therefore, there has been scepticism concerning the ability of tyrosine kinase inhibitors to selectively act on tumour cells, and discussion about safety and side-effects arising from the potential to also act on normal cells. This has been less of an issue in the last few years, as it has been learned that normal cells have multiple, redundant, signalling pathways, but that diseased cells usually have one primary pathway causing the altered signalling.

A second issue that has been widely debated is the potential for general toxicity of tyrosine kinase inhibitors that were designed to be competitive with ATP. Two arguments have fuelled this debate. First, kinases are required for normal cellular functions, such as proliferation, migration, metabolism and survival. The catalytic domains of protein kinases are highly conserved and it was reasoned that compounds must bind similarly into the binding pocket of all ATP-utilising enzymes and, thus, could not be expected to be specific. Therefore, ATP-competitive inhibitors that work on kinases in diseased cells would interfere with the activity of other crucial enzymes in non-cancerous cells and tissues. Secondly, the concentration of ATP inside the cell is sufficiently high and the binding affinity (Km) for ATP is sufficiently low that it would be impossible to identify inhibitors that could overcome both of these properties. Indeed, the design of early kinase inhibitors was based on the structure of phosphotyrosines (tyrphostins). These were designed to be competitive for substrate rather than ATP.

The above has led to the general belief that it would be very difficult, if not impossible, to develop potent, ATP-competitive, selective tyrosine kinase inhibitors. Several examples now exist of tyrosine kinase inhibitors that are very selective, as discussed earlier. Quinazolines have been shown to be competitive for ATP binding to the EGF receptor [165]. It is likely that quinolines and quinoxalines also compete with ATP. It is unclear how such similar compounds can be specific for different receptors. As new kinases and compounds are co-crystallised, the fine differences in receptor specificity may be better understood and allow for the development of new compounds through computer modelling and drug design. For example, crystallisation studies confirmed the binding of SU5402 in the ATP-site, yet it is an example of a relatively selective tyrosine kinase inhibitor with activity on the FGF receptor and the VEGF receptor, Flk-1 [188].

Most small molecule inhibitors currently in clinical development were purposely designed to be very specific. In comparison to conventional chemotherapeutic agents, they had better toxicological profiles in animal models and their safety is now being studied in man. If their safety is confirmed, it is likely that less selective tyrosine kinase inhibitors that have activity on more than one kinase, such as the substituted pyrimidines or indolinones, will be tested as well.

It is important to point out that the long-term consequences of inhibiting tyrosine kinases have not been studied at this time. Given their role in development, it is likely, at the very least, that tyrosine kinase inhibitors would have effects on developing embryos and thus might be contraindicated during pregnancy. This is supported by studies in mice where tyrosine kinases have been genetically altered *via* targeted mutagenesis, resulting in embryonic death.

The future of tyrosine kinase inhibitors as therapeutic agents will be clearer in a few years, when results of the current clinical trials become known. Based on preclinical results, the new generation of tyrosine kinases inhibitors look very promising for the treatment of cancer and other proliferative diseases.

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Exhibit B

CHAPTER 36

Transplantation Immunology

Hugh Auchincloss, Jr., Megan Sykes, and David H. Sachs

Because the transplantation of tissues between members of a species does not occur in nature, a defense against transplantation provides no obvious advantage for the survival of the species. Thus, the allogeneic response (i.e., the immune response to the novel antigens of other members of the same species) probably did not evolve for the purpose of graft rejection, and issues of fundamental immunology cannot be explained on the basis of their importance to allogeneic immunity (1–3). Nevertheless, studies of transplantation biology have contributed significantly to our understanding of fundamental immunology by leading, for example, to the discovery of the major histocompatibility complex (MHC) antigens and by providing the mixed lymphocyte response (MLR) assay for the study of T-cell activation. Now, however, the emphasis has shifted. With the increasing importance of clinical transplantation, the goal is to apply our knowledge of fundamental immunology to the problem of graft rejection.

The common error in many summaries of transplantation immunology is to assume that the field can be understood simply by applying classical immunologic principles to describe the response to this particular set of foreign antigens. Allogeneic responses, however, differ from other immunologic responses in at least two fundamental ways. First, they exhibit extraordinary strength and, probably for that reason, they include unusual types of responses that cannot be detected in classical immunology. Second, they can be stimulated by two different sets of antigen-presenting cells (APCs): those of the donor and those of the recipient. In this chapter, we will emphasize these differences from classical immunology as we describe our current understanding of the several immune responses that cause graft rejection.

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THE ORIGINS OF TRANSPLANTATION IMMUNOLOGY

Early History

The earliest known records of tissue transplantation are those of the Hindu surgeon Sushrutu who reported the use of a flap from a

patient's forehead to repair an amputated nose (4). This procedure was probably practiced by Hindu surgeons as early as 700 B.C. In the 15th century, Italian surgeons began to practice rhinoplasty by means of flaps and extended the donor site to the patient's arm (5). In 1503, one such surgeon reported the first allograft: the grafting of skin from a slave for the reconstruction of the master's nose. A sizable legend grew out of such reports, although obviously unfounded in fact.

Skin grafting became an accepted practice in the late 1800s. Many surgeons, however, did not distinguish between autografts (donor and recipient the same individual) and allografts (donor and recipient of the same species) or even, sometimes, xenografts (donor and recipient of different species). The last of these formed the basis for an extensive practice known as zoografting, in which patients were subjected to grafts from animals ranging from pigs to frogs (6). Billingham points out that no one apparently cared whether the grafted skin "took" or merely promoted healing of the wound (7). The results of these efforts led to a period of confusion in transplantation. Without any clear understanding of the processes involved, surgeons embarked on all sorts of transplants, and a series of operations were reported that we know, from our present understanding of the laws of transplantation, could not possibly have been successful. Dr. Serge Voronoff, for example, attained considerable fame (and fortune) in Europe by developing an unusual grafting procedure in which testicles were transplanted from apes to man in order to restore men to youth and vitality (8).

The transplantation of internal organs awaited the development of techniques for vascular anastomosis. In 1908, Alexis Carrel, one of the pioneers of vascular surgery, reported the results of en bloc allotransplantation of both kidneys in a series of nine cats (9). He was able to obtain up to 25 days of urine output in some cats, but ultimately all of them died. Although other investigators repeated and modified Carrel's experiments, no major advances in prolonging the function of allografts or in understanding the cause for their failure were made for the following three decades.

During this same period, the closely related field of tumor transplantation gained momentum. In 1902, Jensen reported the transplantation of a mouse tumor through 19 successive generations of mice and was able to obtain tumor growth in some 50% of the mice he injected. Furthermore, he showed that mice in whom the tumor grew for a while and then regressed were resistant to subsequent challenge with the same tumor. He also was able to prevent successful tumor grafting by prior treatment with grafts of normal tissues (7).

Although it seems obvious to us now that these experiments provided much of the information essential to an understanding of transplantation immunology, this was not clear at the time. Many investigators still held to the Athrepsia Theory formulated by Paul Erlich in 1906 (10). According to this hypothesis, living tissues required a vital substance specific for each species and provided only by the intact organism. Thus, a transplanted tumor might grow for a while until it used up its supply of this substance. Other investigators who accepted a theory of immunity were nevertheless committed to the idea that they were studying an effect peculiar to tumor tissues. In his Harvey Lecture (11), Sir Peter Medawar summed up the confusion neatly by the statement, "Nearly everyone who supposed that he was using transplantation to study tumors was in fact using tumors to study transplantation."

In 1936, Voronoy, a Russian surgeon, reported the first clinical renal allograft (5). There was apparently a mismatch of blood types and the patient died having demonstrated only minimal renal function. The early postwar years saw reports of attempts at clinical renal homotransplantation from various world centers (12–15). In 1952 the first successful renal transplant was performed in Boston using the kidney of an identical twin (16–19).

One of the important contributions to the understanding of transplantation in this era was the work of Sir Peter Medawar. In 1943, Gibson and Medawar reported their experience with autologous and allogeneic skin grafts on a woman who had suffered extensive third-degree burns (20). The allografts in this case were taken from the patient's sibling, and for clinical reasons they were transplanted in two different stages about a week apart. The authors observed accelerated rejection of the second grafts. Appreciating the possible significance of these observations, Medawar followed them with a series of grafting experiments in rabbits and mice (21,22). By 1945, he was able to conclude that "resistance to homologous grafted skin therefore belongs to the general category of actively acquired immune reactions . . ." (23), thereby establishing the relationship of clinical transplantation to the field of immunology.

History, Principles, and Discoveries of Immunogenetics

Inbred Strains

Rodents have provided an invaluable model for the study of the genetic basis for graft rejection. One of the main features that has made them so valuable is the availability of a large number of inbred strains. Such strains consist of animals that have been produced by sequential pedigreed brother–sister matings for at least 20 generations and which are, therefore, essentially genetically identical. With the exception of the sex chromosomes, chromosomes in such strains are homozygous and therefore produce identical homozygous progeny.

The reason that sequential inbreeding leads to homozygosity is illustrated in Fig. 1. For the sake of simplicity, the first generation illustrated in this figure is indicated as a brother–sister mating in which for any given autosomal locus the alleles being bred will be of the form AB × AB. The more general case of AB × CD also can be analyzed statistically by a similar, although slightly more complicated, mathematical treatment. The ratio of genotypes of the offspring from this breeding are given by the binomial formula (AA:AB:BB = 1:2:1). Thus, as illustrated at generation 2, when a single brother–sister pair is chosen, the chance that both animals will have the genotype AA at the locus in question is $^1/_{16}$. Similarly, the probability that the second generation mating will take the form BB × BB is also $^1/_{16}$. In either of these eventualities, all future generations will be fixed as homozygotes (either AA or BB); therefore, we speak of the locus as being fixed. Thus, the probability of fixation of a given autosomal locus at this generation is $^1/_8$.

For segregation of a large number of independent loci, it is mathematically equivalent to state that the probability of fixing a given locus is $^1/_8$, or that on the average $^1/_8$ of the segregating loci will be fixed. If the locus in question is not fixed during this random breeding, then the chances that it will be fixed at the next breeding are still approximately $^1/_8$ (actually a little larger). In other words, $^1/_8$ of the loci would be expected to fix at the second inbreeding generation, $^1/_8$ of the remaining unfixed loci would be expected to fix at the next generation, and so on. As indicated in Fig. 1, the probability of fixation (P_{fix}) is given by the following formula:

$$P_{fix} = 1 - (^7/_8)^{n-1}$$

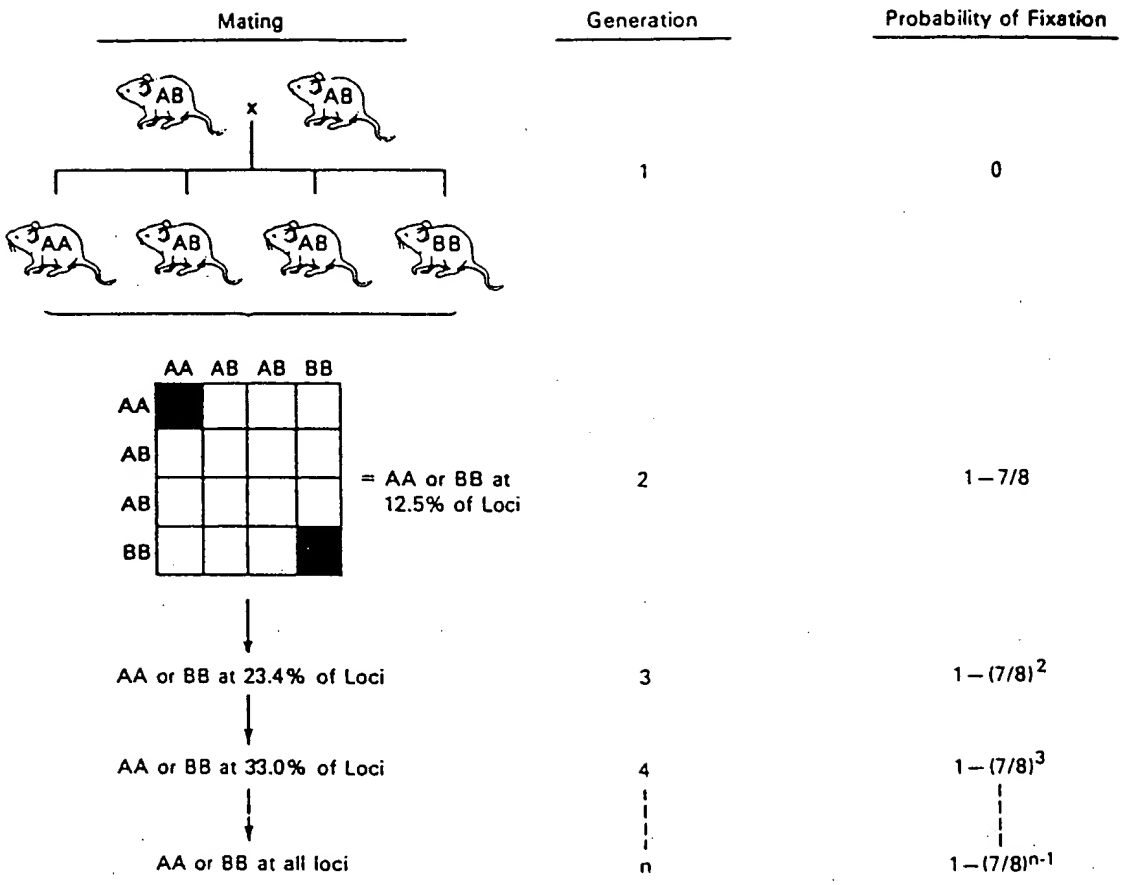


FIG. 1. Breeding scheme for inbred strains. A schematic representation of the inbreeding process. As indicated in the diagram, random selection of a single brother and sister at each generation provides a 1/8 chance for fixation of any autosomal locus as either AA or BB. In other words, 1/8 of the independently segregating loci will be expected to be fixed by each brother–sister mating.

This equation describes a curve that rises asymptotically toward a probability of 100% fixation (Fig. 2). Because genes travel at meiosis in groups rather than individually, there is a finite number of units of genetic information that segregate. Therefore, for practical purposes, one can consider a strain inbred after 20 such brother–sister matings, because at this point there is a very small chance that any locus will not have reached homozygosity. All loci will be of the genotype either AA or BB, and there will no longer be any loci of the heterozygous form AB. The strain so derived is defined as an inbred strain. Hundreds of such well-characterized inbred strains are now available.

During the procedure of sequential brother–sister matings to produce such inbred strains, there are, as expected, numerous cases in which lethal recessive genes become homozygous, leading to the loss of a particular line. However, because a very large number of sequential brother–sister pairs can be started and maintained from a single original breeding pair, it is generally possible to produce at least several inbred strains from the breeding of two outbred animals. If, for example, one sets up all possible brother–sister matings at the first two or three generations and then selects only a single brother–sister pair for all subsequent generations, one might easily obtain 10 successful inbred strains, even if 90% of the lines started were to succumb to lethal recessives. Because a large number of strains of mice can be housed in a small space, such a project is feasible in this species. Inbred strains also have been produced in several other species, including rats, guinea pigs, and rabbits. However, both space requirements and other genetic features, such as gestation times, age of sexual maturity, and litter size, make production of inbred strains in larger species much more difficult.

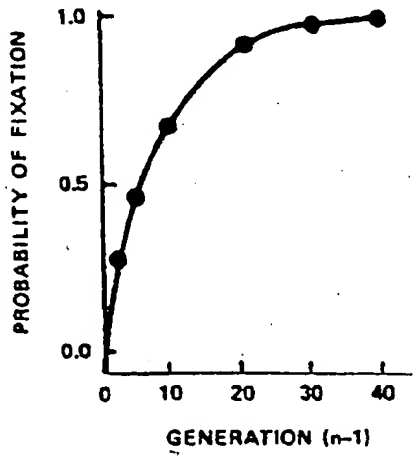


FIG. 2. Probability of fixation curve. The approach to fixation of all loci is asymptotic, given by the formula $P = 1 - (7/8)^{n-1}$ (see Fig. 1).

Several factors may mitigate against the generation of truly inbred animals. One such factor is known as forced heterozygosity. This situation arises when both possible alleles at a given locus are recessive lethals such that only heterozygotes are viable. In this case, the locus in question, as well as loci closely linked to this locus, will be maintained in a heterozygous state. This situation, although theoretically possible, has apparently been encountered only rarely during the production of inbred strains. A more common problem in obtaining true homozygosity is that of mutation, which is of course a continuously occurring phenomenon that cannot be avoided. The average mutation frequency for mammalian genes has been estimated to be approximately 10^{-6} per locus per meiosis. Because there are more than 10^6 genetic loci in mammalian organisms, one would expect at least one mutation to occur somewhere in the genome at every generation. Although one cannot avoid this source of reintroduction of heterozygosity, one can assure that such heterozygosity, once introduced, will not remain for very long by continuing to maintain a brother–sister pedigreed mating scheme for the reference line of any inbred strain. As indicated in Fig. 1, such a scheme will ensure that any mutation that occurs will either be lost or be fixed as a homozygous allele by this procedure. In order to assure that inbred lines stay inbred, therefore, pedigreed reference lines for each inbred strain must be maintained. A single brother–sister mating is chosen at each generation, and all other animals of the strain are bred from this pair or its progeny. Animals used for experiments in large numbers are bred in expansion and production colonies but should not be more than a few generations away from the reference pedigreed line.

If a particular inbred strain is maintained in two different colonies, the pedigreed reference lines will accumulate different mutations. The lines will therefore be said to drift from each other. If proper sequential brother–sister mating is performed in both colonies, each line will remain truly inbred, although eventually the two lines will be distinct at a number of genetic loci. Lines maintained separately are therefore called sublines and are designated by a series of letters following the strain designation, which indicate the origin and location of the pedigreed reference line. Thus, for example, the C3H/HeJ and C3H/HeN lines are two different sublines of the C3H strain. Both were originally maintained by Heston (He), one subline then being maintained at the Jackson Laboratory (C3H/HeJ) and the other at the National Institutes of Health (C3H/HeN). Although these strains are still quite similar for many properties, there are already several known differences between them, such as the responsiveness of their lymphocytes to lipopolysaccharide. Often differences between sublines are first detected when results from one laboratory are found difficult to reproduce in another.

Genetic Principles Governing Tissue Transplantation: The "Laws of Transplantation"

The earliest strains of inbred mice examined by geneticists had been produced for commercial rather than experimental purposes. Mouse fanciers in Europe and Japan had for many years attempted to maintain a variety of desirable characteristics in their mouse lines, such as coat color and behavioral patterns, and in selecting for such traits they had essentially inbred their mouse strains. In the early 1900s it was noted by tumor biologists that tumors arising in such animals could frequently be transplanted successfully to other animals of the same line, whereas this was usually impossible in outbred animals. Little and colleagues then studied this phenomenon systematically, in the process producing and characterizing a large number of inbred strains of mice (24).

In summarizing the results of these studies of tumor grafting in mice, Little described what have since been called the five laws of transplantation (Table 1). These are not really laws but rather a set of apparently confusing observations in which the capacity for graft rejection exists in the parental generation, is lost in the F1 generation, but is regained in the F2 generation in most cases. For those attempting to identify the genetic basis for tumor (graft) acceptance, this pattern did not appear in keeping with Mendelian genetics. Little's remarkable insight was to reconcile these observations with the classical Mendelian principles by suggesting the true fundamental principle of graft rejection and by identifying the genetic basis for the unusual outcomes (25,26). His fundamental principle was that recipients would reject grafts if the donor expressed a product of any histocompatibility (tissue compatibility) locus that was not expressed by the recipient (a principle that is now second nature to any student of transplantation immunology). His explanation for the unusual inheritance pattern was to suggest, first, that there must be codominant expression of the histocompatibility genes, and second, that there must be a relatively large number of histocompatibility loci. Under these conditions, members of the F1 generation would express both parental alleles at all histocompatiblity loci (and thus would fail to reject grafts from parental, F2, or subsequent generations), and members of the F2 generation would be unlikely to express all of the products of histocompatibility genes that are expressed by either parental generation (and thus would usually reject parental allografts).

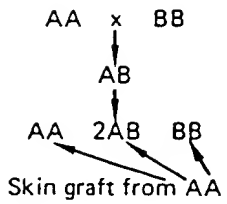
Estimating the Number of Histocompatibility Genes

Given the availability of inbred strains and the genetic principles discussed above, one can experimentally determine the number of histocompatibility loci by which any two inbred strains differ. One breeds a large F2 population between these strains and then transplants tissues from one of the parental strains to all of the F2 offspring, measuring the fraction of grafts that survive. As illustrated in Fig. 3, if the two strains were to differ at only one histocompatibility locus, one would predict that $^3/_4$ of the grafts would survive. If, however, the two strains differed by two independently segregating histocompatibility loci, then one would predict that $(^3/_4)^2$ or $^9/_{16}$ of the grafts would survive because of the $^3/_4$ of animals accepting the graft due to histocompatibility at the first locus, only $^3/_4$ would be expected to be histocompatible for the second locus. Similarly, if there were n loci by which these two strains differed, one would expect $(^3/_4)^n$ to be the fraction of surviving grafts.

TABLE 1. *The laws of transplantation*

1. Transplants within inbred strains will succeed.
2. Transplants between inbred strains will fail.
3. Transplants from a member of an inbred parental strain to an F1 offspring will succeed, but those in the reverse direction will fail.
4. Transplants from F2 and all subsequent generations to F1 animals will succeed.
5. Transplants from inbred parental strains to the F2 generation will usually, but not always, fail.

Modified from ref. 774.



No. Loci	Expected Survivals
1	3/4
2	$(3/4)^2$
.	.
.	.
n	$(3/4)^n$

FIG. 3. Estimating the number of histocompatibility loci. Given two inbred strains, the number of independently segregating histocompatibility loci can be determined by skin grafting to the F2 generation.

When experiments designed to determine the number of histocompatibility loci were first performed, tumor grafts were used. The number of loci detected was between four and 10, depending on the particular parental strains chosen and the tumor used for transplantation. Subsequently these experiments were repeated using skin grafts as the challenging transplant. In this case, numbers for n as high as 30 to 50 have been reported (27–30). Because there are only 20 chromosome pairs in the mouse genome, these larger numbers imply that many chromosomes carry more than one histocompatibility locus.

Producing Congenic Strains: Identifying the MHC

There are thus a very large number of histocompatibility loci, each encoding a cell protein capable of contributing to rejection of a graft. However, in addition to Little's insight that there were multiple histocompatibility loci, the genetic principles he identified also suggested the process for breeding mice that would generate strains differing from one another genetically at only a single histocompatibility locus. This process, pursued especially by Snell at the Jackson Laboratories, involved the production of congenic strains (inbred strains that differ from one another at only one independently segregating genetic locus) using the rejection of parental skin grafts as the trait used to select successive matings (31). The resulting congenic strains were therefore called congenic-resistant strains because they resisted engraftment of tissues from one another. In the course of producing numerous congenic-resistant strains, it became apparent that one histocompatibility locus could be distinguished from all the others by the speed with which it caused skin graft rejection. This is now called the MHC. All of the other 30 to 50 histocompatibility loci have since been called minor histocompatibility loci. There are now a large number of H-2 congenic-resistant strains of mice available (Table 2), as well as some that isolate minor histocompatiblity loci, and some rat congenic-resistant strains.

One of the most useful breeding schemes to produce congenic-resistant lines is illustrated in Fig. 4. Starting with two inbred strains, labeled strain A and strain B, the objective is to obtain a strain that will share its entire genome with strain A except for the major histocompatibility locus H-2, which will be derived from strain B. The end product will be designated as strain A.B. Accord-

TABLE 2. *List of H-2 congenic resistant strains*

Strain	H-2 haplotype	Origin of background	MHC MHC
A	a	A	—
A.BY	b	A	Brackyury
A.CA	f	A	Caracal
A.SW	s	A	Swiss
BALB/c	d	BALB/c	BALB/c
BALB.B	b	BALB/c	C57BL/10
BALB.K	k	BALB/c	C3H
B6.AKR-H-2^k	k	C57BL/6	AKR
B6.SJL	s	C57BL/6	SJL
B10	b	C57BL/10	C57BL/10
B10.A	a	C57BL/10	A
B10.D2	d	C57BL/10	DBA/2
B10.M	f	C57BL/10	Outbred
B10.BR	k	C57BL/10	C57BR
B10.SM	v	C57BL/10	SM
B10.RIII	r	C57BL/10	RIII
B10.PL	u	C57BL/10	PL/J
C3H	k	C3H	C3H
C3H.SW	b	C3H	Swiss
C3H.JK	j	C3H	JK
C3H.NB	p	C3H	NB
D1.C	d	DBA/1	BALB/c
D1.LP	b	DBA/1	LP
LP.RIII	r	LP	RIII

ing to the cross-intercross scheme illustrated in Fig. 4, the two inbred strains are first crossed to produce an F1 generation. Because, as described above, both inbred strains can be presumed to be homozygous at all autosomal loci, all loci of the F1 generation will be heterozygous (ab). These F1 animals are then intercrossed to produce an F2 generation. The distribution of alleles at all autosomal loci in this generation follows the binomial expansion. At any locus, one fourth of the animals would be expected to be of genotype bb. A skin graft or tumor graft from strain A is next placed onto all of the F2 offspring. Animals that reject the graft must be of genotype bb for at least one histocompatibility locus. Obviously, because there are many histocompatibility loci, most animals at this generation will reject the graft. However, if only animals rejecting vigorously are chosen, and if numerous such animals are selected, then one can be reasonably certain to have selected bb homozygotes at the H-2 locus by this procedure.

The process is next repeated by mating rejectors back to strain A animals. For selected loci, therefore, the offspring once again are heterozygous. At all other, nonselected loci, offspring will have a 50% probability of being homozygous for aa alleles or of being heterozygous ab. Obviously, therefore, approximately half of the nonselected genetic information is caused by this process to revert to the inbred strain A type. Once again, these animals are intercrossed to produce the expected F2 distribution for selected loci. Another tissue graft from strain A is performed, and again rejecting animals are selected. The fraction of animals rejecting grafts vigorously at this generation will be smaller than it was at the previous generation. Once again, by selecting only vigorous rejectors, one will assure the selection of the bb homozygote at the H-2 locus. A cross to strain A is again performed, again producing the expected ab heterozygotes at the selected locus or loci. This time,

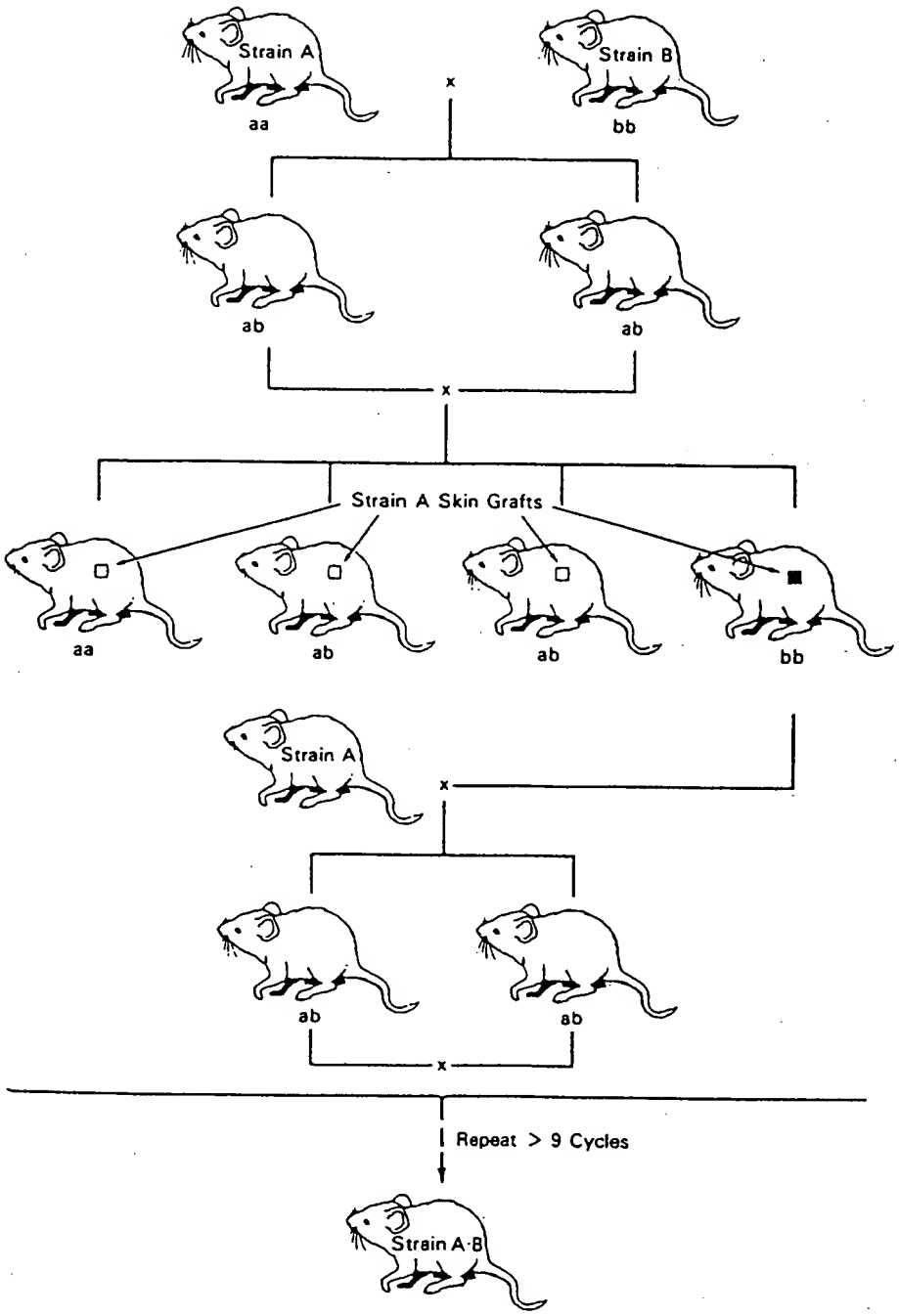


FIG. 4. Schematic representation for production of a congenic line (A.B). Illustrated is the intercross-backgross system, in which selection for histocompatibility genes is performed by skin grafting progeny of each intercross generation and selecting animals capable of rejecting grafts from the strain A parent.

however, the chances that any nonselected locus will still be heterozygous have decreased to 25%.

This process of crossing, intercrossing, and selecting by graft rejection is repeated sequentially. By the time nine cycles have been completed, one would expect there to be only one histocompatibility locus still segregating, so that only 25% of the intercross offspring should be capable of rejecting the graft. Assuming that vigorous rejection has been demanded throughout, one can be relatively certain that the selected locus will be H-2. In addition, the chances that any other nonselected locus will still be heterozygous rather than having reverted to the homozygous aa genotype will have fallen to less than 0.2%. Stated another way, more than 99.8% of nonselected loci will be expected to be identical to their counterparts in strain A. A male and a female homozygote from the final intercross are selected and used to establish a pedigreed inbred congenic resistant line A.B.

Because mammalian genes are transferred as linked units in chromosomes, this process will always lead to the retention of a

variable amount of bb genetic information at genes closely linked to the locus being selected. However, as described below, the occurrence of recombination during intercrossing generations also leads to fixation of the aa genotype at loci on the same chromosome as the MHC (chromosome 17 in mice), but at a variable distance from H-2. For practical purposes, animals that have been through at least nine cycles of such selected breeding are considered to be congenic.

As indicated in Table 2, there are now a large number of H-2 congenic mouse strains available on a variety of backgrounds. In general, the names of each of these strains follow the rule A.B, with Strain A being the background strain used in the production of the congenic, and strain B being the other parental strain from which the alternate allele at H-2 was selected. All of the early inbred mouse strains were assigned a small letter designation to represent the particular constellation of alleles that they possessed at genes in the MHC. This small letter designation is often called the haplotype designation, as indicated in Table 2. Thus, for example, strain C57BL/10 is assigned the haplotype designation H-2b and strain DBA/2 the haplotype designation H-2d. The shorthand designation for C57BL/10 is B10 and that for DBA/2 is D2. Thus, the congenic strain B10.D2 represents a congenic-resistant line in which the background is derived from the C57BL/10 and the MHC from the DBA/2. It thus resembles in almost every way the C57BL/10 congenic partner, except that it differs from this partner for all properties controlled by MHC-linked genes. Similarly, the C3H.B10 strain was derived from an initial cross between C3H (H-2k) and C57BL/10 (H-2b).

The formal designation of a congenic-resistant line also includes, in parentheses after the letters, a designation such as (18M), distinguishing different congenic lines derived from the same cross. Because a large number of congenic-resistant lines have been developed in which histocompatibility loci other than H-2 have been transferred to the same background, these numbers are often included to distinguish different lines. However, for most purposes when one is describing an MHC congenic, one does not need to include its suffix. Thus, B10.D2 is a generally acceptable designation for the H-2 congenic between C57BL/10 and DBA/2.

Intra-MHC Recombinant Strains: Class I and II Antigens

As can be seen in Fig. 4, every alternate generation in this mating scheme involves the crossing of animals heterozygous at H-2. Whenever heterozygotes are bred, there is always a possibility of recombination between autosomal chromosomes at meiosis. During the production of congenic lines, such recombination will tend to decrease the amount of linked genetic information carried into the congenic from the H-2 source. Therefore, the more backcrosses a particular congenic line has been subjected to, the closer will be the boundaries on either side of H-2 at which the chromosome reverts to the background strain. Because it soon became apparent that the MHC was in fact made up of multiple loci, there was also the possibility for recombination within H-2 to occur during such crosses. Indeed, it was through the detection and characterization of such recombinants that the linkage map of H-2 was constructed. It is instructive to

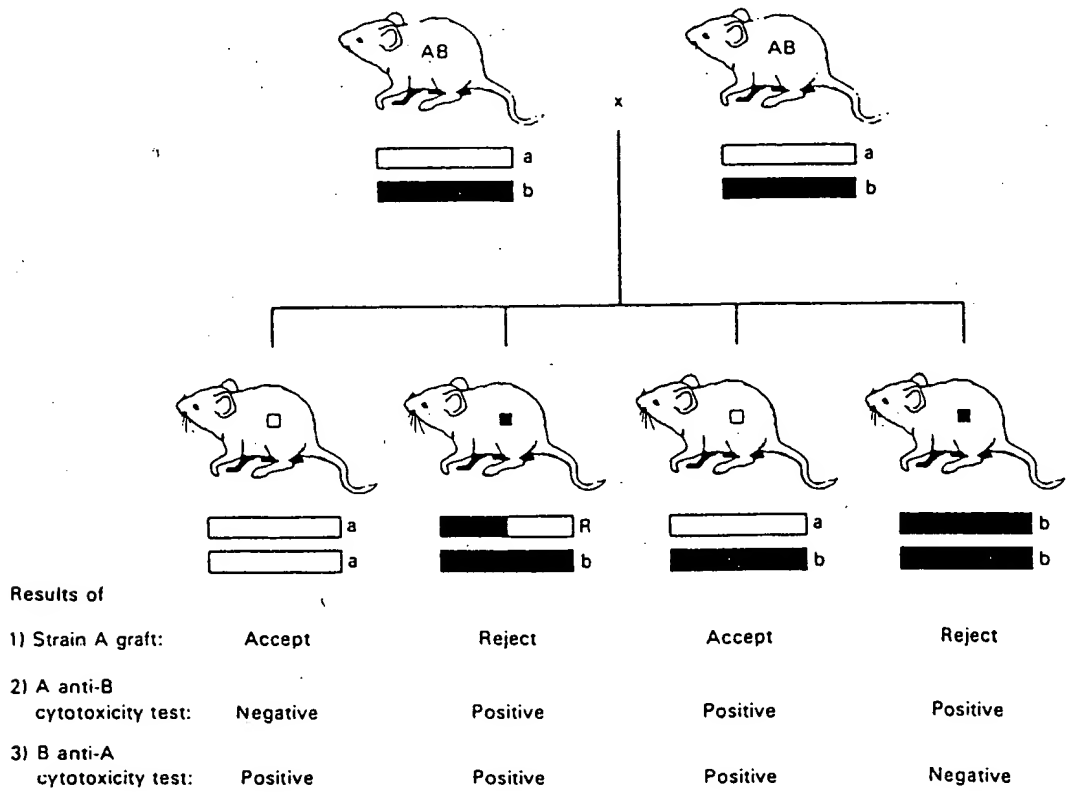


FIG. 5. Occurrence of intra-MHC recombination during the production of a congenic line. Illustrated is the occurrence of a recombination between H-2^a and H-2^b from one of the parents, leading to a new recombinant haplotype in one of the offspring.

examine how such recombinants would be detected and used in determining the genetic fine structure of the H-2 locus.

In order to detect a recombination event, one must examine the intercross or backcross progeny for more than one of the distinguishing features of the MHC described in the subsequent section of this chapter. This is because one can only detect the occurrence of a recombination event if the two properties examined do not behave comparably in the progeny. Thus, during the production of the hypothetical congenic resistant strain A.B, a recombination such as that illustrated in Fig. 5 might occur. In this case, let us assume that progeny are being examined both for ability to reject vigorously a strain A skin graft (i.e., genotype bb for a histocompatibility locus) and for presence of a or b gene products on lymphocyte surfaces as detected by a complement-mediated cytotoxic assay. The antisera used might be strain A anti-strain B (detecting products of bb) and strain B anti-strain A (detecting products of aa).

As seen in Fig. 5, an intra–H-2 recombinant event might lead to an animal that would type as bb by the skin graft analysis because it lacks a part of the H-2 complex that encodes products capable of causing skin graft rejection, but nevertheless type positively with both A anti-B and B anti-A antisera, suggesting an ab genotype. Such an animal would certainly not satisfy the requirement for an eventual A.B congenic line, so that other rejectors would be used for further crossing to produce the desired congenic. However, such an animal might be selected as a putative recombinant and backcrossed further to strain A to produce a congenic recombinant line designated A.B(1R). The next such putative recombinant found would be called A.B(2R), etc. In this way, a series of congenic lines might be obtained, each differing from the background strain A at the MHC, and from each other by different points of recombination within the MHC.

Fortunately, mouse geneticists were aware of this possibility and saved numerous recombinants during the production of H-2 congenic lines. Thus, for example, there are now a series of recombinants between strain C57BL/10(H-2b) and A/WySn(H-2a) which were isolated by Stimpfling during production of the B10.A CR line and which have provided a great deal of information on the genetic fine structure of the H-2 complex (32). Strains B10.A(2R) and B10.A(4R), for example, have been used to map a variety of immune response genes within the MHC. Table 3 lists many of the most useful congenic recombinant strains now available and their known or presumed points of recombination. Among the most important contributions that came from the study of intra-MHC recombinant strains was the progressive understanding that the loci within the MHC encoded two general types of MHC antigens, now referred to as class I and class II MHC antigens.

DONOR ANTIGENS RESPONSIBLE FOR GRAFT REJECTION

Major Histocompatibility Antigens

As discussed above, the genetic analysis of graft rejection indicated that the antigens encoded within the MHC are of particular importance in graft rejection. Table 4 summarizes important aspects of the MHC antigens that are worth emphasizing in this chapter on transplantation. A much more detailed description of their structure and function can be found in Chapter 8.

Basic Features of MHC Antigens

Class I and Class II Antigens

Different loci within the complex encode two general types of MHC antigens, today called class I and class II antigens. Over the years the distinction between these two classes has been based on several different criteria; thus, the terminology applied to them has varied. Originally the class I antigens were identified most easily by serologic techniques, and they were therefore named *serologically defined* (SD) antigens. Class II antigens, however, were not originally detected by antibody responses but by proliferative responses of allogeneic lymphocytes. Class II antigens were therefore called *lymphocyte defined* (LD) antigens (33). Subsequently, serologic identification of class II antigens was accomplished and it was recognized that genes responsible for class II antigens were tightly linked to the I region of the mouse MHC (34). Thus, for a time, these antigens were called I region–associated (Ia) antigens. Among other differences, class I and class II MHC antigens evoke allogeneic responses that differ in both character and magnitude, as will be discussed below.

TABLE 3. *List of H-2 recombinant strains*

Recombinant interval haplotypes	Parental haplotypes	Haplotype designation	K A E S D	Presence of additional recombinant site	Strain bearing recombinant
K-A	b/m	bq1	b/k k k q	Yes	B10.MBR
	s/a1	t1	s/k k k d	Yes	A.TL
A-E	a/b	h4	k k/b b b	No	B10.A(4R)
	b/a	i5	b b/k d d	Yes	B10.A(5R)
	b/a	i3	b b/k d d	Yes	B10.A(3R)
E-S	k/d	a	k k k/d d	No	A, B10.A
S-D	d/b	g	d d d d/b	No	HTG, B10.HTG
	d/k	o2	d d d d/k	No	C3H.OH
	a/b	h1,h2	k k k d/b	Yes	B10.A(2R)
	k/q	m	k k k k/q	No	AKR.M, B10.AKM
	q/a	y2	q q q q/d	No	B10.T(6R)
	s/a	t2	s s s s/d	No	A.TH

Congenic recombinant haplotypes available from The Jackson Laboratory.

Note that many of the recombinants involve at least one haplotype already containing a point of recombination. These are indicated by "Yes" and are listed only under the recombinant interval representing the most recent recombination in

TABLE 4. *Summary of the MHC*

Class I antigens	Single polymorphic chain
	Three domains: alpha 1,2,3
	MW: 45,000
	Associated with Beta 2 microglobulin
	A, B, and C loci in humans
	Expressed on all tissues and cells
Class II antigens	Two polymorphic chains: alpha and beta
	Each with two domains: alpha 1 and 2; Beta 1 and 2
	MW: 33,000 and 28,000
	DP, DQ, and DR loci in humans
	Expressed on macrophages, dendritic cells and B cells; vascular endothelium; activated human T cells

Polymorphism

The MHC antigens exhibit extraordinary polymorphism. This polymorphism presumably provides an advantage to members of the species by ensuring a broad capacity to present the peptides of, and thus respond to, a large number of foreign antigens. The high degree of polymorphism has important consequences for transplantation. The large number of alleles encoded by each locus combined with the presence of at least six individual loci in the MHC in humans make the likelihood of achieving identity for MHC antigens in two unrelated humans extremely small. Some have estimated this probability at one in a million, although the true probability varies enormously depending on an individual's genetic background.

Tissue Distribution

The tissue distribution of the two types of MHC antigens is not identical. Class I antigens are present on all nucleated cells of the body, but they may be sparsely represented on some types of cells, including certain APCs (35,36). Class II MHC antigens are more selective in their distribution (37). They are especially frequent on macrophages, dendritic cells, and B-lymphocytes. They may be present on other lymphoid cells under some circumstances, as well as on vascular endothelium. Their expression on some tissues of the body is not constant and varies according to several stimuli (38). Finally, the tissue distribution of class II MHC antigens is not the same in all species. One of the important distinctions between rodents and many larger species is the lack of expression of class II antigens on the vascular endothelium and other cell populations in rodents, whereas pigs, monkeys, and humans do express class II antigens on these tissues (39).

Physiologic Function of MHC Antigens

MHC antigens are called histocompatibility antigens because of their powerful role in causing graft rejection, yet they did not evolve in nature to prevent tissue grafting. Although the name serves to emphasize the historical importance of transplantation in the discovery of the MHC, the essential role of MHC antigens is now understood to involve the presentation of peptides of foreign antigens to responding T cells (see Chapter 9).

The Importance of MHC Antigens in Alloreactivity

Alloreactivity is the immune response to foreign antigens of other members of the same species. MHC antigens are exceptionally important in stimulating alloreactive responses, both *in vivo* and *in vitro*.

Vigorous Graft Rejection

Allogeneic MHC antigens are the most important antigens responsible for causing graft rejection. Their discovery depended largely on this feature because early experiments showed that mouse skin grafts differing only in their MHC antigens were typically rejected in 8 to 10 days, whereas grafts differing by only a single minor histocompatibility antigen were typically rejected in three or more weeks. Subsequent experiments have confirmed the importance of MHC antigens for other types of grafts. In pigs, primarily vascularized organs such as the kidney may survive indefinitely in some cases, even without immunosuppression, if all of their MHC antigens are matched, whereas MHC-mismatched kidneys are always rejected within 2 weeks (40).

However, the clear evidence for the importance of MHC antigens in causing graft rejection generally depends on there being disparities of both class I and II antigens together. Thus, there are examples in mice of skin grafts that have only class I or only class II MHC antigen disparities that are not rejected at all (41). Furthermore, the importance of MHC antigen matching becomes harder to detect, especially for skin graft survival, when comparing MHC–antigen mismatched grafts with grafts differing in multiple minor histocompatibility antigens.

Primary In Vitro *MLR and CML*

Allogeneic MHC antigens also stimulate an extraordinarily strong T-cell response *in vitro*. This strength is manifested partly by the ability to achieve primary *in vitro* cell-mediated responses to allogeneic MHC antigens, whereas *in vitro* responses to nominal antigens, such as ovalbumin, generally require *in vivo* priming. The greater strength also can be measured by the higher precursor frequency of alloreactive T cells compared with that for other foreign antigens presented in association with self MHC molecules. T cells reactive with an allogeneic MHC determinant may represent as many as 2% of the total T-cell population, whereas T cells reactive with an exogenous protein generally represent only approximately one in 10,000 of the same T-cell pool (42,43). This difference, representing at least two orders of magnitude, is not necessarily the result of a multiplicity of determinants on allo-MHC antigens because the same findings are obtained when precursor frequencies are measured for a mutant MHC antigen varying from the responder by only a single amino acid.

Explanations for the Strong Response to Allogeneic MHC Antigens

Originally, efforts to explain the strength of the immune response stimulated by allogeneic MHC antigens focused on possible physiologic benefits of a strong alloreactive response. For example, some considered the possibility that alloreactivity might be helpful in terminating pregnancy at parturition or that it might help prevent the spread of infectious diseases between individuals.

However, as the true physiologic function of MHC antigens has become better understood, most immunologists have concluded that the strong response to allogeneic MHC antigens is not physiologic, but rather an accidental occurrence that depends on two important features: first, that allogeneic MHC antigens are almost unique among foreign proteins in being able to stimulate an immune response without first being processed into peptides for presentation by self MHC molecules, and second, that because of this feature they can be recognized on allogeneic rather than just on self APCs.

Direct Recognition of Allogeneic MHC Antigens

All theoretical explanations for the extraordinary strength of alloreactivity are based on the unusual feature that T cells can recognize allogeneic MHC antigens without the usual requirement that peptides of these antigens be processed and presented by self MHC molecules. Transplantation immunologists refer to this special type of recognition as direct recognition of allogeneic MHC antigens. The capacity for direct recognition is believed to result from the similarity of the determinants formed by allo-MHC antigens with those created by the presentation of foreign peptides by self MHC antigens. In short-hand terminology, this has been referred to as "Allo = Self + X" (44,45). The evidence supporting this cross-reactive property comes from studies of T-cell clones that are specific for peptide antigens presented by self MHC molecules, but that also recognize allogeneic MHC antigens directly (44–46). Because the T-cell repertoire is selected in the thymus to recognize modified self MHC antigens preferentially (47), it therefore also includes large numbers of receptors capable of recognizing allo-MHC antigens directly.

Although it is widely agreed that direct recognition of allo-MHC antigens is an important component of the strength of alloreactivity, the mere presence of T cells that can respond directly to allo-MHC antigens does not explain why they should be present in higher frequency than the physiologically relevant T cells that respond to modified self MHC antigens. In the same short-hand terminology, the existence of T cells capable of recognizing allo-MHC antigens directly does not, in itself, predict "Allo >> Self + X."

The Strength of Direct Alloreactivity

Three different, but not mutually exclusive, hypotheses have been proposed to explain the high frequency of alloreactive T cells: (a) a genetic bias favoring T-cell receptor genes that are specific for MHC antigens, (b) a greater density of individual allogeneic MHC determinants on the surface of allogeneic APCs, and (c) a greater frequency of different allogeneic MHC determinants on the donor APCs.

Jerne was the first to propose that the genes that encode T-cell receptors might be maintained according to their ability to confer reactivity with the MHC antigens of the species (48). If so, then after the thymus removed self-reactive T cells, the mature T-cell repertoire would include a high frequency of cells reactive with all other MHC antigens. Jerne's hypothesis was proposed before immunologists had learned about associative recognition and positive thymic selection, but his theory became even more attractive in light of these considerations. Because the thymus only selects T cells with some degree of MHC reactivity, a T-cell receptor gene pool that encodes a broad range of specificities (as is the case for B cells) would produce many useless precursors. A narrower pool of T-cell receptor genes, however, as suggested by Jerne's hypothesis, would allow for more efficient thymic selection. There is some evidence to support Jerne's hypothesis (49–55), although the selection of mature T cells within the thymus appears nonetheless to be extremely inefficient (56).

The second explanation for strong alloreactivity, sometimes called the determinant density hypothesis, considers the difference in the expression of nominal antigens, presented as peptides by self MHC molecules on self APCs, and the expression of allogeneic MHC molecules on allogeneic APCs (57). As illustrated in Fig. 6A, the density of nominal antigen determinants expressed by a self APC would be quite low (because most MHC antigens present other peptides), whereas the density of an allogeneic MHC determinant on allogeneic APCs would be very high (because every MHC antigen would represent a foreign determinant). According to this hypothesis there might not really be a higher precursor frequency of alloreactive T cells, but they would appear to exist in larger numbers because the more powerful stimulus of an allogeneic APC would activate many T cells with relatively low affinities.

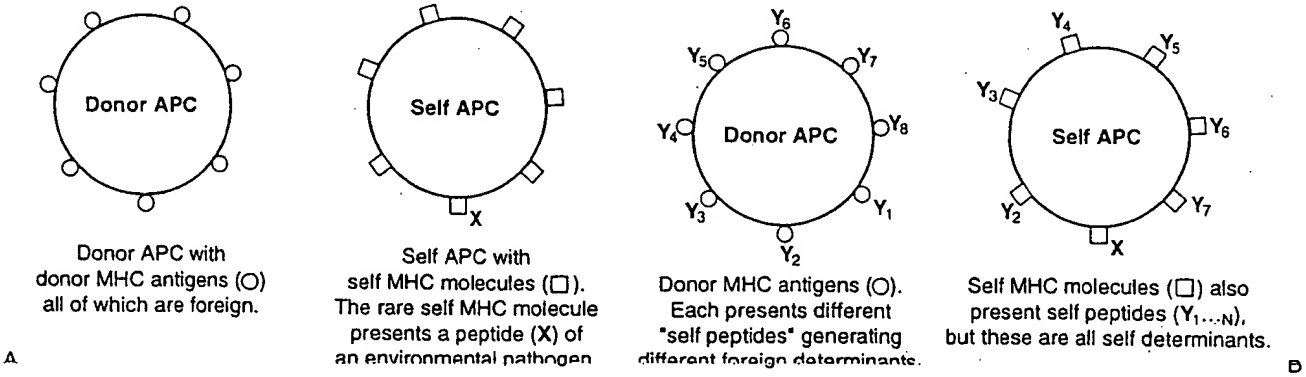


FIG. 6. A: Determinant density hypothesis. Donor APC with donor MHC antigens (O), all of which are foreign (left). Self APC with self MHC molecules (□). The rate at which self MHC molecules present a peptide (x) of an environmental pathogen is very low (right). **B:** Determinant frequency hypothesis. Donor MHC antigens (O), each present different self peptides generating different foreign determinants (left). Self MHC molecules (□) also present self peptides ($Y^{1\cdots n}$), but these are all self determinants.

The third explanation for alloreactivity, sometimes referred to as the determinant frequency hypothesis, was developed on the basis of the idea that T cells specific for allogeneic MHC antigens might be influenced by the peptides presented by these MHC molecules (58). If the MHC molecules on self APCs often present peptides of self proteins (say $X_{1, 2,} \ldots n$), then allogeneic MHC antigens would also present peptides of allogeneic self proteins (e.g., Allo + X_1, Allo + X_2,. . . Allo + X_n") (Fig. 6B). In some cases, the self peptides presented by self or allogeneic MHC molecules might be identical, but the peptides of self proteins presented by allogeneic MHC also might differ from those presented on a self APC. In either case, however, the set of determinants represented by "Self + $X_{1 \ldots n}$" would differ from that represented by "Allo + $X_{1 \ldots n}$." T cells responsive to self peptides on self APCs (Self + X_1, Self + X_2, etc.) are eliminated by the induction of self tolerance, leaving only the rare self MHC molecule, presenting a peptide of a nominal antigen, to stimulate an immune response. On the other hand, self tolerance would not affect the response to the many self peptides on allogeneic APCs (Allo + X_1, Allo + X_2, etc.). Thus, the determinant frequency hypothesis suggests that alloreactive T cells really are more frequent because each allogeneic MHC antigen generates a large number of different foreign determinants.

Choosing between the determinant density and frequency hypotheses depends on the degree to which alloreactive T cells are influenced by the peptides presented by allo-MHC molecules. Although there is some evidence that alloreactive T cells can recognize determinants that are not influenced by peptide presentation (59–61), most evidence suggests that they generally do see "Allo + X" (62–67). Thus, the available information tends to support the determinant frequency hypothesis, although no formal proof of this conclusion is available.

The finding that alloreactivity is so strong often generates confusion in light of the discussion of T-lymphocyte development in Chapter 11. There it was pointed out that the process of positive selection in the thymus generates a T-cell repertoire that is strongly biased toward recognition of peptides presented by self MHC molecules and against the recognition of peptides presented by allogeneic MHC molecules. This would seem to suggest that the response to allogeneic MHC antigens ought to be weak, not strong. However, this confusion occurs only if one forgets that the experiments demonstrating the principles of positive selection could only be performed after T-cell alloreactivity to a particular set of foreign MHC antigens was first eliminated. Under these circumstances, an individual "A" who was tolerant to self and tolerant to "B," whose T-cell repertoire developed in an "A" thymus, would develop T cells capable of recognizing "A + X" much more efficiently than "B + X." However, under ordinary circumstances, an individual "A" who was tolerant only to self, whose T-cell repertoire developed in an "A" thymus, would develop T cells capable of recognizing "A + X" and "B + X," but would also be capable of recognizing "B without X," even in the absence of *in vivo* priming. Thus, the recognition of "B + X" would be uninterpretable in these experiments. Therefore, the phenomenon of positive selection represents the enrichment of T-cell receptors capable of seeing modified MHC antigens after those receptors with strong affinity for the same MHC antigens plus self peptides have been removed. Alloreactivity can occur despite the influence of positive selection because negative selection never occurs for the vast majority of T cells recognizing allogeneic MHC antigens.

Minor Histocompatibility Antigens

The experimental process that initially defined the "major" histocompatibility complex similarly defined the "minor" loci by the slower rejection caused by their antigens. As understanding of MHC antigens increased, however, it became apparent that the separation between major and minor antigens could not depend on the speed of graft rejection alone. Class I or II MHC antigens alone, for example, do not necessarily cause rapid skin graft destruction in mice, whereas the combination of several minor histocompatibility discrepancies may bring about rejection as rapidly as a whole MHC difference (68,69). Thus, the identification of a major histocompatiblity antigen depends in part on the location of the genes encoding the molecule and in part on the well-characterized structure of both class I and class II antigens (see Chapter 8). For example, Qa and Tla antigens are generally considered class I–like products because of their structure, even though they are weak transplantation antigens in terms of rejection. Thus, minor histocompatibility antigens are those capable of causing cell-mediated graft rejection, but which lack the structural characteristics of MHC products (70). This definition of minor histocompatibility antigens does not include all non-MHC alloantigens, but rather focuses on those capable of eliciting a T-cell immune response. Other glycoproteins, such as blood group antigens, which can cause rejection through B-cell responses, are considered below.

For a long time investigators tended to assume that the minor histocompatibility antigens were other allelic cell surface proteins, similar in nature if not in strength to the MHC antigens. We now recognize that this is not the case. The minor histocompatibility antigens, defined on the basis of cell-mediated rejection, are peptides of donor proteins that are presented by MHC molecules (71–78). Thus, the minor histocompatiblity antigens are analogous to nominal foreign antigens, the peptide fragments of which are presented by MHC molecules to evoke a T-cell response. Of course, individuals are tolerant to the peptides derived from their own proteins and can only respond to the peptides of another individual's proteins that have allelic variation, i.e., polymorphism.

During the past several years, some of the peptides representing minor histocompatibility antigens have been isolated (76–78) and, in several cases, the proteins from which they are derived have been identified. As expected, these proteins are not surface glycoproteins, but are instead intracellular proteins such as nuclear transcription factors. Presumably any cellular protein with allelic variation could function as a minor histocompatibility antigen as long as it contains a peptide expressing that allelic variation that is capable of being presented by an MHC antigen in an immunogenic form.

The notion that the minor antigens are peptides recognized in association with MHC molecules has explained many of the features of these antigens that were known, but poorly understood, for a long time (79,80). First, it is difficult, if not impossible, to detect humoral responses to minor antigens. This is probably because most minor histocompatibility antigens come from intracellular proteins and, thus, even if an antibody response did occur, we would not know how to detect it without knowing the protein. Second, minor antigens do not stimulate a primary *in vitro* cell-mediated response, whereas MHC antigens evoke a powerful primary response in both MLR and CML assays. This is in keeping with the general difficulty in detecting *in vitro* T-cell responses to peptides of nominal antigens unless *in vivo* priming has occurred. Third, the recognition of minor antigens is MHC restricted, i.e., secondary

responses require that the minor antigens be presented in association with the same MHC molecules as during the primary exposure (71–75). This would be expected for any antigen that evokes a T-cell response by the presentation of its peptides in the cleft of an MHC molecule. Fourth, when multiple minor antigen discrepancies exist, the immune response to one of these antigens often predominates in a phenomenon known as immunodominance (81–86). This is not due to weak recipient responsiveness to some of the minor antigens because slight changes in the donor–recipient combination sometimes produce strong responses to antigens that evoked weak or no responses before. This phenomenon may be due to competition between peptides of different minor antigens for presentation by MHC molecules.

Although this discussion of minor histocompatibility antigens has emphasized general conclusions, it is based on studies of the responses to individual minor antigens. Several studies have been reported using a variety of congenic-resistant strains that were generated on the basis of weak rejection in order to isolate minor histocompatibility loci (e.g., H-1, H-3, H-41, or H-42) (87–90). In addition, one of the most thoroughly studied minor antigens has been the H-Y antigen, encoded on the Y chromosome, that is therefore expressed only by males of a given species (91–93). There is no reproducible antibody response to this antigen, primary *in vitro* cellular responses cannot be obtained, and secondary *in vitro* cell-mediated responses are MHC restricted. In addition, analysis of the anti–H-Y response has shown that (a) some strains can generate this response whereas others cannot, (b) the immune response genes determining responsiveness are encoded both within and outside the MHC, and (c) the rejection of grafts on the basis of the H-Y antigen alone requires that the antigen generate both helper determinants, recognized by $CD4^+$ cells in association with class II MHC antigens, and cytotoxic determinants, recognized by $CD8^+$ cells in association with class I antigens. This last feature suggests that to be identified as a minor histocompatibility antigen, a protein or perhaps a combination of proteins, probably has to generate at least two different peptide fragments that show allelic variation (94).

Other Antigens of Potential Importance

The minor histocompatibility antigens, defined by their ability to evoke cell-mediated rejection, do not account for all of the non-MHC antigens that can elicit transplantation rejection. Several other groups of antigens also should be considered.

Superantigens

Superantigens share the feature with MHC antigens that they can stimulate primary *in vitro* T-cell proliferative responses and activate an unusually high proportion of the T-cell repertoire. However, these antigens are not presented as peptides in the binding groove of MHC molecules, but instead bind to distinct regions of class II MHC molecules and engage nonvariable portions of Vβ components of the T cell receptor, rather than the hypervariable regions that recognize peptides. Furthermore, it does not appear that endogenous superantigens can serve as transplantation antigens, perhaps because they are not expressed by endothelial cells or parenchymal cells of most tissues (95–99). Thus, superantigens cannot be classified as transplantation antigens of any type.

Tissue-Specific Antigens

There is evidence that some peptides presented by MHC molecules may be derived from proteins with limited tissue distribution (100–102). For example, T-cell clones specific for allogeneic cells of one type do not always recognize cells of a different type from the same individual (103).

The only well-described tissue-specific antigen that causes graft rejection is that for skin, referred to as the Sk antigen (100). Because it was identified on the basis of T cell–mediated responses, this antigen most likely represents a peptide, derived from a protein expressed only in skin, that is presented by an MHC molecule. This tissue-specific protein differs from the minor antigens, however, in that it need not necessarily show allelic variation because the determinant formed by "Allo + X_{sk}" (where X_{sk} is a peptide derived from the skin-specific protein) would be different from that formed by "Self + X_{sk}." Hence, T cells can be tolerant to the skin-specific antigen expressed on their own tissues, but responsive to this same antigen of a different individual. The tissue specificity of this antigen occurs because "Allo + X_{sk}" is expressed only by donor skin and not by other donor tissues.

The existence of tissue-specific antigens has importance in several ways. First, *in vitro* assays to measure T-cell responsiveness to donor antigens may be misleading when they use donor lymphohematopoietic cells as the stimulating population if the actual T-cell response is specific for donor tissue–specific antigens. Second, the need to develop self-tolerance to tissue-specific antigens emphasizes that the induction of tolerance might not be accomplished entirely in the thymus by a central process (104). Finally, the existence of tissue-specific antigens suggests that transplantation tolerance induced by one set of donor cells might not always induce complete tolerance to donor cells of a different sort.

Endothelial Glycoproteins

Blood Group Antigens

The blood group antigens do not evoke cell-mediated responses and hence are not classified as minor histocompatibility antigens. They are expressed on many types of cells and, importantly, are present on vascular endothelium where they may serve as the targets for an antibody-mediated attack on blood vessels.

Blood group antigens were identified because of their importance in transfusions (105). They represent the effects of glycosylation enzymes such that A and B individuals each express their respective antigen but O individuals have neither. The natural antibodies that develop against these antigens probably do so as a result of cross-reactions with common carbohydrate determinants of environmental microorganisms as long as the individual does not already express those determinants. Thus, O individuals will develop antibodies to the antigens of A and B donors, whereas A and B individuals will only develop antibodies reactive with antigens from each other, and AB individuals will develop responses to neither. Therefore, O recipients can only receive transfusions from O donors; A and B recipients can receive transfusions from O donors or from individuals sharing their blood type, and AB recipients can receive blood from donors of any blood type. The same rules apply to the transplantation of most primarily vascularized organs in humans because the vascular endothelium expresses ABO antigens (106,107). In addition to the ABO locus, there are other loci determining blood group antigens on erythrocytes, but

these are irrelevant to organ transplantation because they are not expressed on vascular endothelium.

Other Allogeneic Endothelial Glycoproteins

In addition to the well-known blood group antigens, other glycoproteins expressed on the vascular endothelium may serve as targets for humoral responses. Rarely, preformed antibodies to these antigens may give rise to hyperacute rejection of primarily vascularized organs. In addition, antibody responses to endothelial glycoproteins can be detected after kidney transplantation between MHC identical, blood group–matched individuals (108). However, these induced antibodies may not have any role in graft rejection.

Species-Specific Carbohydrate Determinants

Closely analogous to the blood group antigens are the carbohydrate determinants expressed on vascular endothelium that show species specificity. For example, pigs have a glycosyl-transferase enzyme that is not expressed by humans, that glycosylates N-acetyllactosamine to form a gal-α(1,3)α-gal determinant. In humans, a fucosyltransferase generates instead the H substance from the same substrate, leading to blood group O. Preformed or "natural" antibodies are present in human serum that react to the novel pig determinant. Similarly, natural antibodies are present between all but the most closely related species combinations. Like the blood group antibodies, these natural antibodies probably arise from cross-reactions with environmental microorganisms (109,110), and they also cause hyperacute rejection of most primarily vascularized xenogeneic transplants. They also may be recognized by other components of the innate immune system, such as macrophages and NK cells.

The Hh locus

In apparent violation of the laws of transplantation described above, a phenomenon has been described in mice whereby (A × B) F1 offspring can reject bone marrow from parental donors. This phenomenon, as well as the phenomenon of rapid rejection of fully allogeneic marrow, was shown in studies by Cudkowicz and colleagues to be mediated by natural killer (NK) cells (111). However, the identity of what appeared to be recessively inherited transplantation antigens responsible for this rejection could not be determined. Recently, it has become clear that the specificity of NK cell–mediated marrow rejection is due to the expression by NK cells of receptors that recognize specific class I MHC ligands on target cells, and that transmit an inhibitory signal to the NK cell upon such recognition. The absence of some self class I molecules on, for example, AA parental hematopoietic cells permits subsets of (A × B)F1 NK cells that recognize class I molecules from the B parent to destroy AA cells. The nature of NK-cell recognition of class I MHC is discussed in detail in Chapter 17.

COMPONENTS OF THE IMMUNE SYSTEM INVOLVED IN GRAFT REJECTION

Antigen-Presenting Cells

Types of APCs

The role of specialized APCs in the process of immune activation is discussed elsewhere in this textbook (see Chapters 9,15, and 16). The critical role of APCs in graft rejection is best exemplified by the prolonged survival of some types of grafts when APCs of the donor have been eliminated (112–116).

Several types of cells have antigen-presenting capability, including dendritic cells, macrophages, and activated B cells (117–121). In addition, several organ-specific cell populations, such as Kuppfer cells in the liver and Langerhans' cells in the skin are probably subpopulations of dendritic cells. Not all APCs are equally effective, and those of the dendritic lineage are the most potent on a per-cell basis (122). All of the "professional" APCs are derived from bone marrow progenitors.

Antigen-presenting cells express MHC class II antigens constitutively, and the level of class II antigen expression can be further increased by various lymphokines, including interferon (IFN)-γ and tumor necrosis factor (TNF)-α (123–125). Some APCs may express relatively low levels of MHC class I antigens, which might serve to protect these crucial cells from destruction by the activated immune response before they can provide their full helper function (35).

An important feature of transplantation immunology is that the APCs responsible for T-cell activation may potentially originate from either the donor graft or from the recipient. The types of APCs in each case are unlikely to be the same because those from the donor will generally be the tissue-specific APCs (such as Langerhans' cells), whereas those from the recipient will generally be those associated with lymphoid tissues. Furthermore, the MHC antigens expressed by the two different sets of APCs often will be different and, thus, the specificities of the T cells stimulated by the two different sets of APCs will generally differ. Unless there is matching of MHC antigens between donor and recipient, only the determinants expressed on donor APCs will also be expressed by parenchymal cells of the graft.

Direct Versus Indirect Antigen Presentation

Because the distinction between the two potential sets of APCs is so important in describing and understanding the mechanisms of graft rejection, transplantation immunologists have developed a terminology to describe the two potential processes of T-cell sensitization. The direct pathway refers to antigen presentation by APCs derived from the donor graft, whereas the indirect pathway refers to donor antigen presentation by recipient APCs (75,126,127). Of course, indirect recognition corresponds to the form of presentation used in classical immunology; thus, the term "indirect" unfortunately seems to suggest that this is not the physiologic process for stimulating an immune response. Actually, direct recognition is the nonphysiologic pathway.

The use of the terms "direct presentation" and "indirect presentation" sometimes becomes confusing. The definition is based on which set of APCs (donor versus recipient) is involved in T-cell activation, not on the mechanisms of antigen presentation. Therefore, although indirect presentation must clearly involve peptide processing, direct presentation also may do so. For example, if peptides of donor MHC class I molecules are presented by donor class II antigens (or even other donor class I antigens), this would still represent direct presentation, even though it involves antigen processing and peptide presentation. In addition, the terms may be confusing in cases where the donor and recipient share some MHC antigens. Under these circumstances, the determinants formed by direct and indirect presentation may be identical. Nonetheless, the distinction between donor and recipient APC stimulation of T cells

is still valid and may be important. Finally, the term "indirect" sensitization, which refers to the process of T-cell stimulation by an APC, can be confused with terms describing effector mechanisms. One frequently encounters discussions of possible indirect effector mechanisms of graft rejection, referring to cases in which T cells appear to recognize determinants expressed only on recipient APCs but not in the donor graft. If such effector mechanisms exist, they would be stimulated by the indirect pathway (afferent arm of the immune response) and they would also mediate graft destruction (efferent arm) indirectly because the graft would lack the target antigen. The two events are obviously related, but the word "indirect" applies to two different phases of the T-cell response.

Trafficking of APCs After Transplantation

Because APCs are the critical element in stimulating immune responses, an important issue in the regulation of transplant rejection and tolerance is which APCs are available and where they are located. Studies in mice show that changes in the location of both donor and recipient APCs take place almost immediately after transplantation. Donor APCs begin to migrate from the graft to the recipient lymphoid compartments, finding their way to both draining lymph nodes and the spleen of the recipient (122). Simultaneously, bone marrow–derived APCs from the recipient begin entering the graft and gradually replace the donor APCs. The time required for this change probably varies for different organs. In the case of murine skin grafts, the replacement of donor by recipient APCs seems to require many months, whereas the shift may occur over weeks in the case of pig kidney and human liver grafts (128–132).

Anatomic Sites of Sensitization

Although activation of T cells generally involves contact with APCs, and allogeneic APCs are especially powerful stimulators, it does not necessarily follow that cell sensitization occurs within the donor graft. Because donor APCs migrate to recipient lymphoid compartments, sensitization may occur primarily in these locations. Experiments by Barker and others have suggested that draining lymph nodes are the primary site of sensitization for skin graft rejection (133–136). They showed that skin grafts on vascular pedicles that had been deprived of lymphatic drainage failed to undergo rejection and failed to prime the recipient against donor antigens. These grafts, however, were susceptible to rejection if the recipient was sensitized by normal skin grafts placed concurrently.

The notion that allogeneic sensitization occurs primarily in draining lymph nodes is in keeping with the principles of fundamental immunology. Naive T cells generally traffic in the lymphoid circulation, waiting for foreign antigens to be concentrated there (137). Only T cells that have been previously activated are allowed to migrate into the nonlymphoid tissues, seeking the source of the antigen challenge (138,139). On the other hand, some memory T cells that were previously activated by "Self + X" determinants may cross-react with allogeneic determinants of a new graft. Thus, it is not surprising that there is also evidence suggesting that activation, or perhaps reactivation, of alloreactive T cells can occur within grafts, especially when they are primarily-vascularized and express donor endothelial cells (135,140,141).

B Cells and Antibodies

Preformed Antibodies

Anti-donor antibodies that are present before transplantation are extremely important in causing rejection of many types of primarily vascularized organ transplants. If they are present in sufficient quantity and recognize determinants expressed on vascular endothelium, preformed antibodies can cause hyperacute rejection. The preformed antibodies that do this are of two general types: natural antibodies and antibodies generated by previous exposure to transplantation antigens.

Natural Antibodies

Natural antibodies include those directed at blood group antigens and species-specific carbohydrate determinants. Their existence does not require previous exposure to transplanted tissues because they are probably generated in response to carbohydrate determinants on microorganisms. They tend to be of the immunoglobulin (Ig)M class, although IgG isotypes also may occur. Their presence is generally thought to be T cell independent, and their receptors are often in, or near, germline configuration.

Preformed Antibodies from Prior Sensitization

Recipients also may express antidonor antibodies if they have been previously exposed to cells expressing the donor antigens. This can occur by prior blood transfusion, as a result of pregnancy, or from previous organ transplantation. The antibodies formed in this way are usually IgG in isotype, are directed at protein rather than carbohydrate determinants (usually against MHC antigens), and have much higher binding affinities than the natural antibodies. Probably because of the density of MHC antigen expression and high affinity of the antibodies, lower titers of these antibodies cause organ damage more consistently than even higher titers of natural antibodies.

Induced Antibodies

After transplantation, new antibodies may be formed to the novel determinants expressed on donor tissue. Often this antibody response is directed at MHC antigens, although antibody responses to other molecules with allelic variation also may occur, especially if the recipient is repeatedly immunized. Induced antibody responses start with IgM antibody formation and then convert to IgG production in a T cell–dependent fashion.

In most cases induced antibodies are not responsible for acute graft rejection, either because they appear too late, after T-cell responses have already caused rejection, or because they appear so slowly, in the face of immunosuppression, that they fail to cause acute graft destruction. However, there are exceptions to this rule that are best demonstrated by primarily vascularized xenotransplants between closely-related species (142–144). In these cases, the induced antibody response occurs especially rapidly and causes an accelerated form of rejection targeted at the vascular endothelium. A similar form of rejection occurs only rarely in allogeneic combinations, when antidonor antibodies appear unusually early, probably reflecting prior sensitization. Induced antibodies after transplantation may play a role in chronic graft rejection. This

process also involves injury primarily to the donor vessels, but occurs over a much longer period of time.

T Cells

Most allograft rejection involves T cell–mediated responses. The particular importance of T cells has been confirmed experimentally by the demonstration that athymic mice accept tissue grafts indefinitely from other members of the same species, and usually from members of other species (145). Furthermore, repopulation of these mice with purified T cells reconstitutes their ability to reject grafts (41,146,147). In humans, the use of reagents that specifically block T-cell responses, as well as the correlation of their effectiveness with their ability to eliminate T cells, supports the central role of T cells in graft rejection (148).

Because there is no phenotypic marker that correlates precisely with the function of particular T-cell subsets, it has been difficult to determine the exact role of the various subsets that participate in graft rejection. Nonetheless, a distinction between helper and effector functions can be made and is important in understanding the process. In the case of T-dependent B-cell responses, the role of T cells as helper cells for B cells that produce alloantibodies has been demonstrated well (149). There have also been *in vivo* experiments that have indicated a distinction between helper and effector T-cell functions for cell-mediated rejection. For example, there are particular cases of skin grafts that are not rejected unless simultaneous grafts that express both the antigenic determinants of the first graft and additional determinants are placed elsewhere on the same recipient (41,150). The rejection of both grafts under these circumstances indicates that a T-cell effector mechanism was potentially available for the rejection of the first graft, but that it required an additional T-cell helper function to allow the effector response to occur. These types of experiments have defined the distinction between helper and effector T-cell functions for graft rejection *in vivo*, and they have suggested the terms "helper determinants" and "effector determinants" based on which determinants were expressed on the first or second grafts. Because in these types of experiments the effector determinants have usually been presented by class I antigens, which are likely to stimulate $CD8^+$ cells, whereas the helper determinants have usually been presented by class II antigens, which stimulate $CD4^+$ cells, the results of these experiments have supported the idea that $CD4^+$ T cells often provide help for $CD8^+$ cells, at least in those cases where the two functions reside in separate cell populations.

Other Cells

Natural Killer Cells

Natural killer cells are large granular lymphocytes that lack T-cell receptors and have the ability to mediate cytolysis against certain tumor targets and hematopoietic cells. NK cells also produce a number of proinflammatory cytokines, including TNF-α and IFN-γ. NK cells can be activated and triggered to kill through a number of different cell surface receptors, some of which may still be undefined, and they represent a first line of defense against a variety of microorganisms. It has recently become clear that NK cells of both humans and mice express clonally distributed surface receptors that are capable of recognizing specific class I MHC molecules. These class I receptors, which are type II C lectin membrane proteins in the mouse (Ly49 family) and are either Ig supergene family members (p58/p70) or dimers of CD94 with NKG2 lectins in the human, are referred to as killer cell inhibitory receptors (KIRs). Recognition by a KIR of a class I molecule results in intracellular transmission of an inhibitory signal via an immune receptor tyrosine-based inhibitory motif (ITIM) that interacts with a tyrosine phosphatase and counteracts activating signals transmitted from other cell surface molecules. Recognition of self class I inhibitory ligands is believed to be important in preventing the NK cell from killing normal autologous cells (151,152).

Although the role of NK cells in mediating hybrid resistance and allogeneic marrow rejection is well-established in mice, the amount of resistance mediated by NK cells to allogeneic pluripotent hematopoietic stem cells is limited and can be readily overcome by increasing the dose of donor stem cells administered (153). Furthermore, despite the fact that human NK cells, like those of mice, have class I–dependent recognition mechanisms that inhibit lysis of targets expressing those class I molecules, a role for NK cells in resisting human allogeneic marrow engraftment has not been clearly demonstrated. However, studies in the mouse indicate a greater role for NK cells in resisting xenogeneic marrow (154) than allogeneic marrow engraftment (153,155).

Natural killer cells are also prominent in infiltrates found in rejecting allogeneic organs and sponge allografts. However, there is no clear evidence that NK cells contribute to solid organ allograft rejection. If they do, NK cells must be dependent on T cells because mice lacking T cells are unable to reject nonhematopoietic allografts. Furthermore, whereas bone marrow allografts from class I–deficient donors (β2 microglobulin [β2m] negative) are subject to potent NK-mediated rejection [because these cells cannot trigger inhibitory receptors on host NK cells (156)], $\beta2m^-$ skin grafts are not rejected by $\beta2m^+$ recipients (157). These results are consistent with the likelihood that NK cells do not reject solid tissue allografts.

Inhibitory receptors on NK cells are quite broad in their class I specificity (158), and recognition of even fully allogeneic class I molecules can confer some protection from NK-mediated marrow destruction compared with that observed for cells deficient in class I expression (156,159). Because of the increased disparity of xenogeneic compared with allogeneic MHC molecules, a greater role might be expected for NK cells in rejecting xenografts than allografts. Indeed, NK cells appear to mount greater resistance to xenogeneic than to allogeneic marrow engraftment in mice (154,155).

Consistent with the hypothesis that NK cells are poorly inhibited by xenogeneic compared with allogeneic MHC molecules, NK cells also have been implicated in the accelerated rejection (160) that can destroy solid organ xenografts that have escaped hyperacute rejection. Because one mechanism by which NK cells mediate cytolysis is via antibody-dependent cell-mediated cytotoxicity (ADCC), it is possible that IgG natural antibodies play a significant role in initiating NK cell–mediated rejection. NK cells also release cytokines, such as IFN-γ and TNF-α, that activate macrophages and endothelial cells and induce inflammation (160). In addition to failing to receive inhibitory signals from xenogeneic MHC molecules, NK cells also may be activated by direct recognition of xenogeneic determinants. For example, it has recently been suggested that lectins on the surface of human NK cells can activate cytolysis when xenogeneic carbohydrate determinants such as α1,3 gal are recognized (161).

Recent studies in allogeneic bone marrow chimeras have suggested that bone marrow engraftment induces a state of tolerance

among NK cells so that both donor and host class I MHC antigens are regarded as self by the NK cells. Similar to the T-cell repertoire, the development of a self-tolerant NK-cell repertoire is adaptively acquired (159,162–164) and is determined by the expression of particular members of the family of molecules (Ly-49 molecules in the murine system) that recognize class I antigens (162). This capacity of hematopoietic cells to tolerize host NK cells may prove to be an important advantage of the mixed chimerism approach for xenotransplantation.

T Cells that Express NK Cell–Associated Markers

In recent years, a subset of murine T cells that express NK cell–associated phenotypic surface markers has been defined. It appears that some of these cells are thymus-dependent (165) and others thymus-independent (166,167) and that they produce a variety of cytokines, including IFN-γ and interleukin (IL)-4 (168). These cells appear to recognize the nonclassical class I molecule CD1 (169) and have been suggested as a possible initial source of IL-4 that can drive T-helper type 2 responses in naive T cells. The cells can be either $CD4^+CD8^-$ or $CD4^-CD8^-$. Humans appear to have a parallel subset of cells (170,171) that use a similar invariant α chain with restricted Vβ gene usage in their T-cell receptor (172,173). This T-cell subset has been reported to play a role in the phenomenon of hybrid resistance in mice (174).

Monocytes/Macrophages

A role in graft rejection for other nonspecific cellular effectors such as monocytes has been suggested (160), especially in xenograft rejection (175). It is likely that proinflammatory cytokines produced by activated monocytes and macrophages, such as IL-1 and TNF-α, play a role in endothelial cell activation. Chemoattractants produced by the inflammatory process may partially explain monocyte recruitment.

MECHANISMS OF GRAFT REJECTION

At least four distinct mechanisms that can cause graft rejection have been identified so far, and it is likely that additional mechanisms will be characterized in the future. It is convenient to describe these mechanisms according to the time frame in which they tend to occur in clinical practice, especially because their names (hyperacute rejection, accelerated rejection, acute rejection, and chronic rejection) have a clear temporal distinction. However, it is increasingly possible to characterize these mechanisms according to the cell types and processes involved and, in some cases, they may occur at uncharacteristic times.

Rejection Caused by Preformed Antibodies (Hyperacute Rejection)

Hyperacute rejection is said to occur when a vascularized organ suffers from rejection within minutes to hours after transplantation. The phenomenon is visible and dramatic. Transplanted kidneys that have initially perfused well turn blue and mottled shortly after vascularization is established. Urine output ceases and recovery does not occur. Microscopically, organs show evidence of extensive vascular thrombosis and hemorrhage with little evidence of a mononuclear cell infiltrate (176).

There are several important components involved in the mechanism of hyperacute rejection. First, there are donor endothelial MHC antigens or carbohydrate determinants as described above. Second, there are preformed antibodies that can bind these antigens. Third, the complement and coagulation cascades are activated by the binding of preformed antibodies to the donor antigens. Finally, there are complement regulatory proteins that can modify complement activation, and anticoagulants that can modify the coagulation pathway. The target of the hyperacute rejection process is the donor vascular endothelium.

The interaction of these components leading to hyperacute rejection is diagrammed in Fig. 7. The crucial event in the process is the formation of the membrane attack complex (MAC), made up of C5–9 of the complement cascade (177,178). In allogeneic combinations, this is always initiated by antibody–antigen binding, which activates complement through the classical pathway. In a few xenogeneic combinations, complement activation also can occur through the alternative pathway and thus does not require antibody binding (179). Complement activation is controlled by several regulatory molecules, including complement receptor 1, decay accelerating factor (DAF, CD55), membrane cofactor protein (CD46), and CD59, which act at different stages along the cascade (see Chapter 29). Many of these molecules are produced by the vascular endothelial cells. Because these regulatory proteins prevent unwanted complement activation in the face of low levels of per-

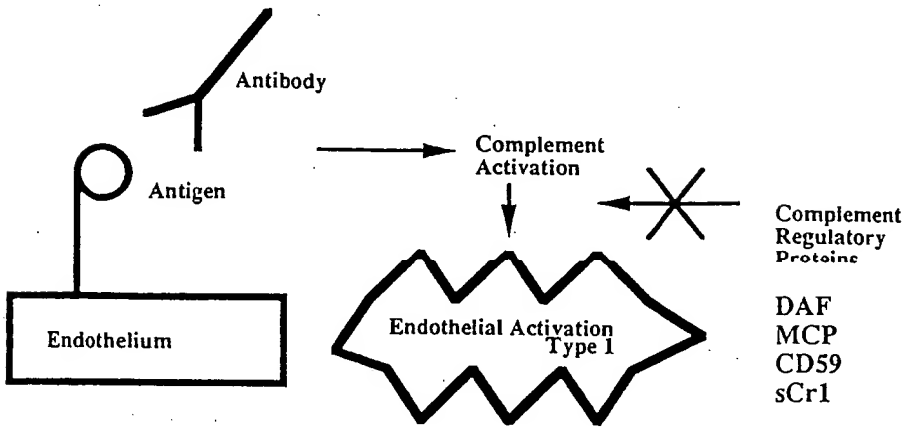


FIG. 7. Schematic representation of hyperacute rejection.

turbation to the system, the initial stimulus for activation must be strong enough to overcome these downregulating molecules. Thus, the titer and avidity of the preformed antibodies must be relatively high. Preformed antibodies directed at MHC antigens almost always accomplish this activation, whereas the lower affinity blood group antibodies lead to hyperacute rejection in only about 25% of cases. One of the reasons that hyperacute rejection is such an important feature in xenogeneic transplantation is that the complement regulatory proteins produced by the donor vascular endothelium of one species do not always function effectively with complement molecules derived from a different species (180). Because of this homologous restriction, lower levels of an initial triggering signal lead to explosive complement activation.

Although the MAC is often thought of as a lytic molecule, its effect on the donor vascular endothelium, even before cell lysis, is to cause endothelial activation (181). This occurs rapidly, before there is time for new gene transcription or protein synthesis, and has been referred to as type I endothelial activation. The two principal manifestations of this activation are cell retraction, leading to gaps between endothelial cells, and the loss of antithrombotic molecules from the endothelium (182). Thus, type I endothelial activation is responsible for the two principal pathologic findings in hyperacute rejection: (a) extravascular hemorrhage and edema and (b) intravascular thrombosis.

There are no known treatments that can stop the process of hyperacute rejection once it has started; thus, it is essential to avoid the circumstances that initiate it. Experimentally, this can be accomplished for relatively short periods of time by administration of cobra venom factor, which depletes complement (183). In clinical practice, this is accomplished by avoiding transplantation in the face of preformed antibodies, both by avoiding blood group antigen disparities and by testing recipients before transplantation to determine whether they have preformed anti-MHC antibodies that react with the donor's MHC antigens. This test is referred to as a cross-match and is usually performed by adding recipient serum to a suspension of donor lymphocytes and measuring cell lysis in the presence of an exogenous source of complement (184). In a small number of cases, allogeneic transplantation in the face of preformed antibodies has been attempted after first removing antidonor antibodies by plasmapheresis (185). This has been successful in some cases involving blood group disparities, but rarely in cases involving preformed anti-MHC antibodies. Discordant xenogeneic transplantation always involves preformed antibodies and thus cannot be accomplished without initial efforts to modify the process of hyperacute rejection.

Not all organs and tissues are equally susceptible to hyperacute rejection. Most primarily vascularized organs, such as kidneys and hearts, are susceptible, but the liver often can survive without hyperacute rejection despite preexisting antidonor antibodies (186–188). It is not clear whether this unusual feature of the liver reflects the large surface area of its vascular endothelium or an intrinsic property of liver endothelial cells. It is possible that because of its anatomic position in the portal circulation, the liver has more powerful mechanisms to prevent endothelial activation resulting from antigen–antibody complexes. Nonetheless, hyperacute rejection of the liver has occurred in some cases, especially involving xenogeneic transplantation, indicating that its resistance to hyperacute rejection is not absolute. The other types of transplants that are resistant to hyperacute rejection are those that do not immediately expose donor vascular endothelium to the recipient's circulation. For example, skin grafts do not suffer hyperacute rejection because their blood vessels are not in communication with those of the recipient until about a week after transplantation (189). After this, large doses of exogenously administered antidonor antibodies can destroy skin grafts through a complement-dependent mechanism (190). Nonetheless, long-term survival of xenogeneic skin grafts has been achieved despite the presence of natural antibodies, suggesting that the threshold for initiating this late antibody-mediated rejection is hard to achieve (191). Fresh pancreatic islets appear to behave like skin grafts, but cultured pancreatic islets (which lose their endothelial components in culture) are probably never susceptible to hyperacute rejection (192–194). Free cellular transplants, such as bone marrow cells or hepatocytes, do not have an endothelium and thus are not susceptible to the mechanisms of hyperacute rejection. However, in many cases these cell transplants do express some of the antigens recognized by preformed antibodies, and there is evidence indicating that the presence of these antibodies can lead to resistance to engraftment (195). Although this resistance can be overcome by transplanting larger numbers of cells, this finding suggests that preformed antibodies can cause cellular graft rejection by mechanisms that are distinct from hyperacute rejection.

Although hyperacute rejection is a dramatic and powerful mechanism of graft rejection, it is rarely encountered in clinical practice. The understanding of its causes, and the use of standard immunologic assays to detect preformed antidonor antibodies, has largely eliminated its occurrence. This is one of the best examples where an understanding of immunology has had an important impact on clinical transplantation.

Early Rejection Caused by Induced Antibodies (Accelerated Rejection)

A second mechanism of rejection, usually caused by antibodies, is almost as infrequent as hyperacute rejection. It occurs as a result of antibodies that are induced rapidly after a transplant is performed. This type of rejection has sometimes been called accelerated rejection because it typically occurs within the first 5 days, but there is no consensus regarding this name. The process is characterized by fibrinoid necrosis of donor arterioles with intravascular thrombosis (196).

Accelerated rejection is rare in allogeneic combinations because it requires that an antibody response occur before the T-cell response that is typically responsible for early rejection episodes. Indeed, in allogeneic combinations, accelerated rejection is sufficiently rare that some investigators have questioned its existence. Other investigators who have studied this mechanism of rejection in xenogeneic combinations have given it several different names, such as acute vascular rejection (197) or delayed xenograft rejection (198), thereby generating confusion about what may be a single process involving endothelial activation occurring later than the type I activation described above. In some xenogeneic cases, accelerated rejection may occur even without an antibody response and may result from endothelial activation by NK cells or other components of the innate immune system.

There are several causes for the difficulty in characterizing accelerated rejection. First, the clinical circumstances are rare in which induced antibodies appear before T cell–mediated rejection. Second, some patients develop antidonor alloantibodies weeks or months after their transplant, but without suffering an acute rejection episode. Finally, it is difficult experimentally to induce a B-

cell response in the absence of T-cell immunity. Thus, it has been hard to prove that an induced B-cell response, rather than an especially vigorous T-cell response, is responsible for a unique rejection mechanism.

Given these difficulties, the best characterization of accelerated rejection has been achieved using primarily vascularized organ transplants from closely related xenogeneic species (142). In these cases, the levels of preformed antibodies are not sufficient to cause hyperacute rejection, but antidonor antibodies appear rapidly (within 3 to 4 days) in association with the onset of rejection (142–144). Vigorous anti-T cell immunosuppression has little effect on this early rejection, whereas immunosuppression with reagents that affect B-cell responses, such as cyclophosphamide, delays its onset until more typical T cell–mediated rejection occurs (142). The two types of immunosuppression together can lead to prolonged graft survival, unless release of the B-cell suppression allows the appearance of antidonor antibodies and the concurrent initiation of rejection (142). The pathology in these cases shows a paucity of lymphocytes infiltrating the donor graft, antibody binding to donor vascular endothelium, and fibrinoid necrosis of the donor vessels.

These studies of concordant xenograft rejection have indicated that the most important feature in accelerated rejection is the early appearance of antidonor antibodies. In fact, these antibodies appear so early, and despite the presence of anti-T cell immunosuppression, that they probably do not represent a primary response to the donor's antigens in most cases. For xenografts, they may represent a rapid increase in the levels of natural antibodies that were present before transplantation, but at undetectable levels. In the case of allografts, low levels of preformed antibodies also exist occasionally as a result of previous exposure to donor MHC antigens, but with the levels having fallen to a point where they are not detected in the standard cross-match. Thus, it is probably the unusual rapidity and perhaps the especially high levels of the antibody response that are critical in causing accelerated rejection.

As in hyperacute rejection, the process of accelerated rejection is usually initiated by antibody binding to antigens on the donor vascular endothelium. In this case, however, the subsequent endothelial changes occur more slowly, allowing time for gene transcription and new protein synthesis. This later form of activation has been called type II endothelial activation (198). Many of its features appear to be mediated by the transcription factor NF-κB, which generates many of the responses associated with inflammation, including the secretion of inflammatory cytokines such as IL-1 and IL-8 and the expression of adhesion molecules such as E-selectin and intercellular adhesion molecule (ICAM)-1 (199). In addition, type II endothelial activation causes the loss of thrombomodulin and other prothrombotic changes (200). Thus, the events following type II endothelial activation are associated with the pathologic changes that occur with accelerated rejection, including the tendency toward intravascular thrombosis and the inflammatory destruction of donor vessels that occurs in the absence of infiltrating lymphocytes.

Just as there are regulatory processes for complement activation, there are regulatory molecules that counter the tendency toward intravascular coagulation and the process of type II endothelial activation. For example, the expression of tissue factor protein inhibitor by vascular endothelium tends to inhibit factor Xa of the clotting cascade (201). In addition, the tendency toward type II endothelial activation is inhibited by the expression of a number of protective molecules, including, bcl-x_L, bcl-2, and A20 (198). Although these are often thought of as antiapoptotic molecules, they also tend to inhibit activation mediated by NF-κB. Just as the regulatory molecules of complement may not function across species differences, so too some of the regulatory molecules involved in type II endothelial activation may show homologous restriction (201). Thus, in addition to the more rapid appearance of antidonor antibodies, loss of regulation also may be responsible for the finding that accelerated rejection is an important aspect of xenogeneic graft rejection (once hyperacute rejection is avoided), whereas it is rarely seen in allografts.

Although vigorous early antibody responses generate type II endothelial activation and accelerated rejection, later antibody responses usually fail to do so. The process that enables transplanted organs to survive in the face of circulating antibodies that can bind endothelial antigens has been called accommodation. In xenogeneic combinations, and some allogeneic combinations with preformed blood group antibodies, accommodation has been achieved by the removal of preformed antibodies for a period of 1 to 2 weeks and the allowance of their slow return after this time. Similarly, resistance to type II endothelial activation has been achieved *in vitro* by pretreatment with low levels of antiendothelial antibodies that are insufficient to trigger activation (202). The achievement of accommodation is associated with increased expression of the antiapoptotic genes described above and with changes in the isotype of the recipient's antibody responses (198).

Although both hyperacute rejection and accelerated rejection occur early after transplantation and depend on antidonor antibodies, there are a number of important differences between the two. One of these is that although hyperacute rejection is primarily mediated by complement activation, accelerated rejection can occur in the absence of complement. On the other hand, accelerated graft rejection may involve different secondary mediators, such as monocytes and macrophages. In xenogeneic combinations (where the inhibition of NK cells by class I molecules is lost), NK cells also may participate in accelerated rejection using antibodies to generate an ADCC response. Possibly NK cells alone can cause type II endothelial activation in xenogeneic combinations, even in the absence of antidonor antibodies, perhaps by triggering through activating lectin molecules that recognize xenogeneic carbohydrate moieties (161).

Another important difference between the two early forms of antibody-mediated rejection involves treatment. Once hyperacute rejection is initiated, there is no known therapy that can stop graft destruction, whereas accelerated rejection can sometimes be reversed by vigorous therapy. This has usually included plasmapheresis to remove antidonor antibodies and treatment with anti–B cell reagents such as cyclophosphamide (142–144). These reagents also may have a direct effect on the donor endothelium, blocking the process of type II endothelial activation. Although treatment of accelerated rejection is possible, it is not always successful. In current clinical practice, this form of humoral rejection may be responsible for many of the relatively few cases in which immunologically mediated graft loss occurs during the first several months after transplantation.

Rejection Caused by T Cells (Acute Rejection)

Although in clinical practice few allogeneic organs suffer either hyperacute or accelerated rejection after careful cross-matching, rejection episodes occurring toward the end of the first week after transplantation are not infrequent, despite the use of immunosup-

pression. These episodes are separable from the humoral rejection processes by the later timing of their occurrence, by the absence in many cases of antidonor antibodies in the recipient, and by the cellular infiltrate usually present in the biopsy. Called acute rejection episodes, most rejection treated by clinicians is of this type. Acute rejection may occur at any time after the first few days following transplantation, but with decreasing frequency after the first 3 months. However, rejection that appears to be similar in mechanisms may occur much later after transplantation, especially if immunosuppressive medication is withdrawn.

Acute rejection of organ allografts is T cell mediated. Therefore, treatment is usually with increased doses of standard immunosuppressive drugs or with antilymphocyte antibodies. These strategies are so likely to be successful that the diagnosis of acute rejection is doubtful if they are not (148).

Because T cell–mediated acute allograft rejection plays such an important role in clinical transplantation, there has been considerable study of the mechanisms involved. Nonetheless, the following discussion will indicate that many important issues regarding this rejection process remain to be resolved. On the other hand, most current clinical therapies to control cell-mediated rejection have been developed in the absence of a thorough understanding of the process but have still been extremely effective in controlling it. Whereas 30 years ago the majority of transplant recipients experienced one, or several, rejection episodes, and only about half of the recipients were able to keep their transplanted organ for a full year, the use of newer immunosuppressive drugs and monoclonal anti-T cell antibodies has changed these numbers considerably. Recent studies have shown that as many as 80% of kidney transplant recipients never experience an episode of acute rejection (203), and it is now rare to lose a transplanted organ to cell-mediated rejection during the first year after transplantation. These impressive results suggest that further analysis of cell-mediated rejection is unlikely to lead to better short-term survival of transplanted organs.

Nonetheless, the experimental study of cell-mediated rejection continues to receive considerable attention for several reasons. First, T cell–mediated responses may contribute to the process of chronic rejection. Second, a better understanding of the mechanisms of T cell–mediated rejection may help to identify assays that would accurately measure the state of a particular recipient's immune responsiveness to their transplanted organ, which therefore might help identify those individuals who needed more or less immunosuppression. More importantly, a better understanding of how T cells cause graft rejection may help in the design of strategies to eliminate this response altogether, by inducing tolerance to the donor antigens. Achievement of this goal would be the ultimate accomplishment of transplantation immunology.

The study of cell-mediated rejection *in vivo* has used four types of experiments. First, there have been the studies of clinical transplants, which are obviously highly relevant and provide frequent tissue for analysis, but which are always performed in the presence of immunosuppression and without the capacity to manipulate important variables. Second, there have been studies of skin grafts or islet transplants using rodents, which provide large amounts of controlled data, but which may not accurately reflect the processes of rejection for primarily vascularized organs. Third, there have been studies of heart transplants and occasionally other types of primarily vascularized organ transplants in rodents, although these types of transplants are too easily accepted compared with similar transplants in human beings. Finally, there have been studies of primarily vascularized organ transplants performed in large animals, such as monkeys or pigs, which have obvious clinical relevance, but which are expensive and difficult to perform in large numbers. The conclusions suggested by these different approaches have not always been the same; thus, the description of the general mechanisms of T cell–mediated rejection is complicated by the need to identify exceptions and features that occur only in special cases.

The Simple, Classical Model of T Cell–Mediated Allograft Rejection

The description above of the donor antigens involved in rejection indicated that allogeneic MHC antigens play a special role in stimulating T-cell responses, especially by direct recognition of these antigens. In addition, the description of the components of the recipient's immune system involved in rejection indicated that APCs play an important role in sensitizing T cells and that sensitized helper T cells lead to the development of an effector mechanism for T cell–mediated graft rejection. Based on these considerations, the simplest model of a T-cell rejection mechanism is that shown in Fig. 8. This model emphasizes the importance of direct recognition of donor class II MHC antigens by recipient $CD4^+$ T cells, which then serve as helper cells for recipient $CD8^+$ cells, which are sensitized by direct recognition of donor class I MHC antigens. The $CD8^+$ cells then provide the effector mechanism for graft rejection based on the direct recognition of parenchymal cells throughout the donor graft that express class I antigens.

A powerful feature of the model shown in Fig. 8 is that it emphasizes that T cell responses to allografts cannot be thought of strictly in terms of the classical mechanisms of T-cell activation described throughout this textbook on fundamental immunology. This is because alloreactivity has the unique feature that donor MHC antigens do not require processing and presentation of their peptides by recipient MHC molecules in order to stimulate a T-cell response. By concentrating on the direct recognition of donor MHC antigens, expressed on donor APCs, the model in Fig. 8 seems to capture the critical features that distinguish allograft rejection from other immune responses, determine its strength, and explain the particular importance of MHC compared with other donor antigens. Nonetheless, as will be described in detail below, the model in Fig. 8 fails to predict the outcome of transplantation experiments under a variety of different experimental conditions. For example, this simple model would predict that the elimination of either $CD4^+$ or $CD8^+$ T cells from the recipient would prevent graft rejection, whereas MHC-disparate skin grafts can be rejected by either subpopulation alone (149,204). This, and many other examples described below, make it clear that the simple model shown in Fig. 8 is inadequate to describe T cell–mediated graft rejection.

There are at least two basic weaknesses with the simple model in Fig. 8. On the one hand, it fails to emphasize sufficiently the importance of direct recognition and the strength of this response. As a result of the high precursor frequency of T cells that respond to allogeneic MHC antigens directly, populations of T cells that ordinarily have minimal significance become functionally important. For example, $CD8^+$ T cells that recognize allogeneic class I antigens directly can generate their own IL-2 and function independently of $CD4^+$ helper cells. On the other hand, the second weakness with the model in Fig. 8 is that this simple model may overemphasize the importance of direct recognition by ignoring the potential contribution of recipient APCs and their capacity to present peptides of donor antigens in the manner of a classical immune response.

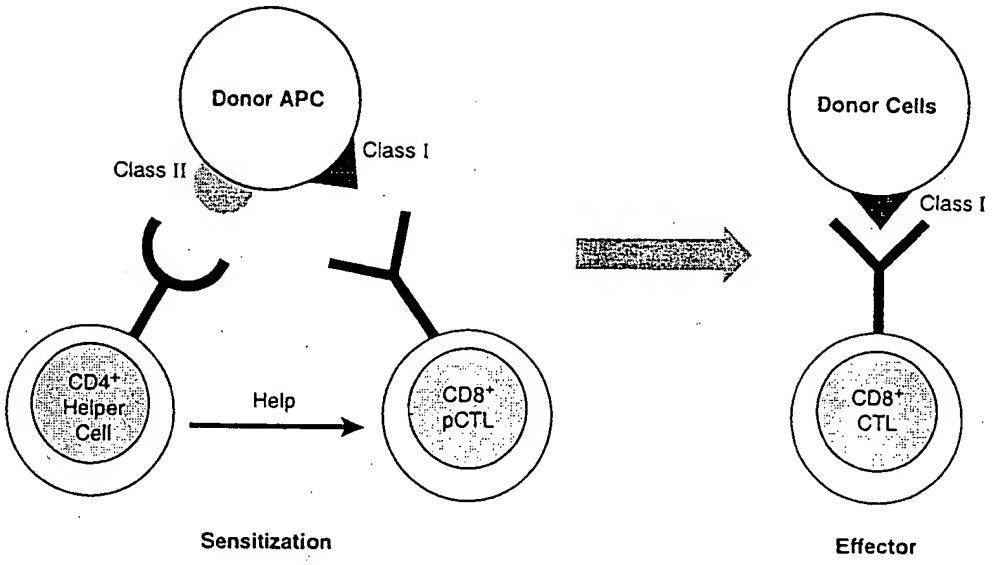


FIG. 8. Simple model of T cell–mediated rejection.

The primary finding from experimental studies of T cell–mediated rejection is that there are multiple ways in which T cells can be sensitized to donor antigens and probably several types of effector mechanisms of graft destruction. Thus, it has become common to talk about pathways of alloreactivity to describe the multiple processes that may be involved. For example, experimental systems have been developed to investigate graft rejection that depends on the indirect pathway alone or that depends on direct recognition by $CD8^+$ cells in the absence of $CD4^+$ cells. This reductionist approach to the analysis of T cell–mediated rejection has become possible because of the availability of antibodies that deplete selected T-cell subpopulations in the recipient, because of mutant and genetically engineered strains of donor mice that express isolated class I or class II antigen disparities, and because of genetically engineered strains of mice that lack the expression of class I or class II antigens. The results of the numerous experiments, both *in vitro* and *in vivo*, that have examined the pathways of alloreactivity are described below, leading to the generation of a more complex model of T cell–mediated graft rejection.

Pathways of Alloreactivity: Helper Responses

Alloreactive Helper Activation Measured In Vitro

The standard *in vitro* assay of helper function in cellular immunity is the MLR. This assay measures proliferation of T cells after allogeneic stimulation. More recently, investigators have found it useful to quantify and characterize the helper response more precisely by measuring the production of particular lymphokines such as IL-2 using biologic assays, enzyme-linked immunosorbent assays, and semiquantitative polymerase chain reaction (PCR). Extensive investigation of the pathways of alloreactive helper activation has been undertaken using these *in vitro* techniques (205–210). A summary of the experimental results is shown in Table 5, which indicates the magnitude of the helper response for each T-cell subpopulation in response to each type of antigenic challenge. It should be noted that in some cases where the response is shown in Table 5 to be absent (based on bulk culture experiments), there have been T-cell clones derived (presumably exceptional cases) that demonstrate this specificity.

The results summarized suggest that there are three main pathways of alloreactive helper activation *in vitro*: $CD4^+$ lymphocytes responding directly to allogeneic class II stimulation, $CD4^+$ lymphocytes responding to peptides of alloantigens presented in association with responder-type class II MHC molecules, and $CD8^+$ lymphocytes responding directly to class I alloantigens. The $CD4^+$ direct response is easily measured. The ability to measure the $CD4^+$ indirect response usually requires *in vivo* priming, although there is an unexpected, weak primary response to peptides of allogeneic MHC antigens presented by self MHC molecules. The ability to measure the $CD8^+$ direct response is often enhanced if IL-2 production rather than just proliferation is measured, especially if an anti–IL-2 receptor antibody is used to prevent IL-2 consumption (207). In addition, detection of the $CD8^+$ direct response requires depletion of the $CD4^+$ population or stimulation with just a class I antigen disparity because helper responses generated by whole MHC differences are generally dominated by $CD4^+$ cells (211). Although $CD8^+$ helper cells can be activated directly by alloantigens *in vitro*, it has been much harder to demonstrate $CD8^+$ helper cells in response to modified self class I antigens. They have not been found in response to TNP-modified self class I molecules (205), but have been detected after *in vivo* priming with virus followed by *in vitro* stimulation with virus-modified class I antigens

TABLE 5. In vitro *pathways of alloreactivity*

	Helper pathways				Cytotoxic pathways	
	Direct		Indirect			
	I	II	I	II	I	II
$CD4^+$	–	++++	–	+++	–	++
$CD8^+$	++	–	±	–	++++	++

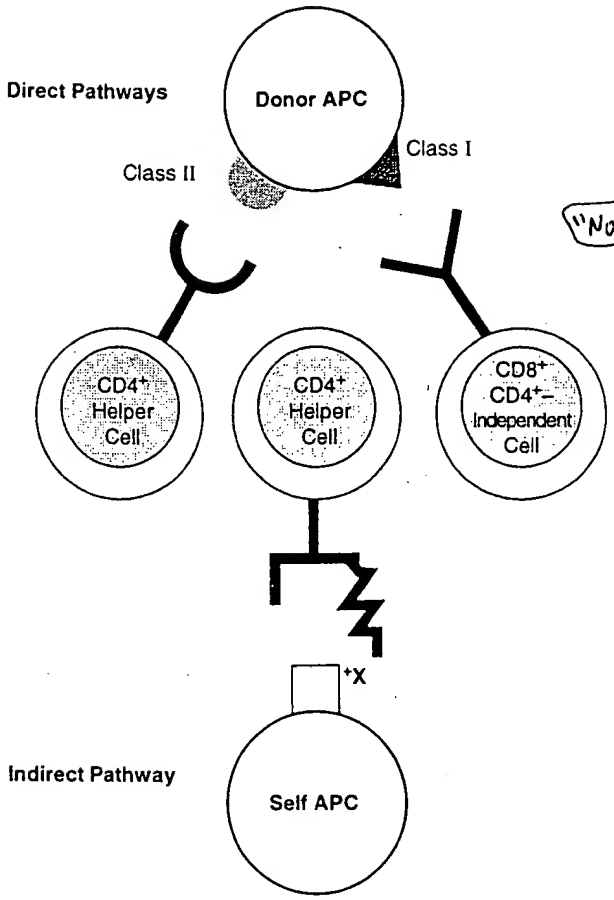


FIG. 9. Complex model of T-cell sensitization pathways.

(212). The $CD8^-$ cells that have helper function in allogeneic responses have been found to have particularly high affinity for class I alloantigens (213).

Alloreactive Helper Activation Measured In Vivo

Two basic approaches have been used to study the pathways of alloreactive helper activation *in vivo*. First, selected T-cell subpopulations have been depleted *in vivo* (149,204,214–221), and second, selected T-cell subpopulations have been transferred into nude or severe combined immunodeficient (SCID) recipients, which themselves lack the T-cell components of graft rejection (41,146,147,217,222–224). In both cases, the approach has been modified by placing grafts with narrow antigenic disparities or by using different types of tissues for transplantation. The results of these studies suggest that all three alloreactive helper pathways detected *in vitro* also can be detected *in vivo*.

$CD4^-$ Direct Activation In Vivo. $CD4^-$ T cells alone can cause rejection of many types of grafts, including those with a class II antigen disparity and those with multiple minor antigen disparities (204,223,225). Thus, $CD4^-$ cells must provide the helper function in these cases. Furthermore, $CD4^-$ cells have been shown to contribute to rejection of almost every type of graft, the only exception being the rejection of some types of class I–only disparate murine skin grafts (41,146). Thus, $CD4^-$ helper function is an important component in vivo in most cases of graft rejection (226).

The role of the direct pathway in stimulating $CD4^+$ cells has been demonstrated using genetically engineered class II knock-out mice as recipients after reconstitution of their $CD4^+$ T-cell population. These mice lack class II antigens on their own APCs and therefore cannot generate an indirect response. Thus, CD4-dependent rejection of grafts by these recipients must reflect direct sensitization of $CD4^+$ cells in vivo (227).

$CD4^+$ Indirect Activation In Vivo. Although indirect $CD4^+$ activation has been easily demonstrated in vitro, it has been difficult to prove that the indirect pathway for $CD4^+$ activation actually contributes to graft rejection in vivo. This experimental difficulty stems from the powerful $CD4^+$ direct response that is almost always present, making it difficult to isolate the effect of indirect activation. Furthermore, some experiments looking for evidence of $CD4^+$ indirect helper activation using class II–matched, class I–mismatched murine skin grafts failed to demonstrate a role for the indirect pathway (41,146). However, accumulating evidence now suggests that indirect activation can contribute to graft rejection:

1. MHC-mismatched APC-depleted endocrine grafts are not rejected, whereas similarly depleted MHC-matched grafts sometimes are, suggesting that an indirect response to determinants expressed on the donor graft may be responsible for rejection (228,229).
2. Xenograft rejection in mice is very dependent on $CD4^+$ function, even though $CD4^+$ direct activation measured *in vitro* is very weak (230).
3. Antibody blocking of recipient class II antigens *in vivo* can sometimes prevent rejection (231).
4. Immunization of recipients with peptides of donor antigens before transplantation can speed subsequent graft rejection (232).
5. Rejection of some grafts lacking class II antigen expression has been shown to be dependent on $CD4^+$ T cells, which were therefore presumably stimulated by modified class II antigens of the recipient (233).
6. Some manipulations that alter the immune response to peptides of donor MHC antigens have been found to prevent rejection of grafts expressing the intact MHC antigens, suggesting that the indirect response to these antigens may be dominant over the direct response in causing graft rejection (234,235).

$CD8^+$ Direct Activation In Vivo. $CD8^+$ T cells alone can reject some types of grafts, including skin grafts that express an MHC class I antigen disparity (41,146,149,236). These results suggest that $CD8^+$ direct activation also can contribute to graft rejection.

Direct $CD8^+$ activation does not appear to be as powerful as direct $CD4^+$ activation because grafts expressing only class I antigen disparities are usually rejected more slowly than class II–disparate grafts, and responses dependent on $CD8^-$ helper responses are more easily suppressed by cyclosporine (237–239). Probably as a result of this weakness, rejection that depends on $CD8^+$ direct activation is influenced by several factors that do not seem to be as important for $CD4^+$ direct activation. First, $CD8^+$ direct activation is dependent on the relative number of donor APCs in a graft (149,233). This may explain why many primarily vascularized grafts that express a class I antigen disparity still require $CD4^+$ cells to initiate rejection. Second, $CD8^+$ helper cells recognize modified self class I antigens poorly (87,205). Therefore, $CD8^+$ direct activation fails to initiate rejection of grafts with only a small number of minor antigen disparities and provides only a weak helper response even when there are a large number of foreign minor antigens.

$CD8^+$ helper cells also differ from $CD4^+$ helper cells in being unable to provide help for other cell populations (240). Apparently the IL-2 produced by these cells is used by the cells themselves as they develop effector function. Therefore, $CD8^+$ helper cells cannot provide help for $CD8^+$ cells with a different specificity and cannot provide help for B-cell antibody responses.

A More Complex Model of the Sensitization Pathways of Alloreactivity

The results of the experiments described above indicate that the simple diagram of alloreactive pathways shown in Fig. 8 is not sufficient and that the more complex model shown in Fig. 9 more accurately depicts the possible routes of sensitization that can initiate graft rejection. Not all of these pathways are available in every case, however, and their availability may change over time, especially because donor APCs are replaced by recipient APCs. Determining the relative importance of the alloreactive pathways is one of the important issues in transplantation immunology at this time.

Which Pathways Are Important in Graft Rejection?

We cannot yet determine precisely the relative importance of the three alloreactive pathways of helper activation of naive T cells in graft rejection. Probably the particular helper activation pathway used depends on the type of graft involved, the antigenic disparity, the time after transplantation, and the previous history of the recipient. In general, the *in vitro* and *in vivo* data suggest that the $CD4^+$ direct pathway is more important than the $CD8^+$ direct pathway unless $CD4^+$ T cells are depleted or class I antigen disparities alone are present. In addition, *in vitro* studies suggest that it is more difficult to suppress either one of the direct pathways than the $CD4^+$ indirect pathway and that it is harder to suppress the $CD4^+$ direct pathway than the $CD8^+$ direct pathway (237,241). Of the various helper pathways, the $CD8^+$ direct response is probably the least important *in vivo* because most studies of rejection, except those using skin grafts, indicate that $CD4^+$ cells are essential for initiating rejection.

The Emerging Emphasis on Indirect Recognition

These results would seem to suggest that although there may be many pathways of alloreactivity, as shown in Fig. 9, the classical $CD4^+$ direct response shown in Fig. 8 is nonetheless the dominant response. However, an important event in transplantation immunology over the past several years has been an increasing tendency to stress the importance of indirect recognition (242–246). This trend has been supported by several different observations. First, it has long been recognized in clinical practice that the amount of immunosuppression needed to achieve prolonged graft survival does not make transplant recipients immunologically crippled. This observation is contrary to the prediction, inherent in Fig. 8, that because of the strength of direct recognition, the immune response leading to graft rejection should be much stronger than any ordinary immune response. Second, a number of investigators have sought to correlate rejection activity in clinical patients with *in vitro* measures of T-cell sensitization. In some cases, they have suggested that an increase in the precursor frequency of T cells responding through the indirect pathway provides the best correlation with clinical events (247–254). Third, recent studies with the class II knock-out mice, in which graft rejection may be studied under conditions where either direct or indirect pathways are available in isolation, have indicated that rejection mediated through the indirect pathway is a powerful event, and that it is often as difficult to control as rejection depending on direct recognition. In some cases, helper responses mediated by indirect recognition even appear to be suppressed by sensitization through the direct pathway (227). Fourth, the recent studies using peptides of allogeneic MHC antigens to suppress the rejection of grafts expressing the intact MHC molecules suggest that manipulations that can only affect the indirect response can have a dominant effect on graft rejection (255–258). These results not only indicate that indirect responses participate in graft rejection, but they also suggest that indirect recognition may be more important than direct recognition. On the other hand, descriptions of graft rejection that emphasize the indirect pathway must deal with several new issues that emerge from this shift in orientation.

Special Problems Associated with Indirect Recognition

Explaining The Importance of MHC Antigen Matching. One of the most attractive features of the classical model shown in Fig. 8 is that it provides a clear basis for explaining the importance of allogeneic MHC antigens in causing graft rejection. As described above, direct recognition of intact allogeneic MHC antigens gives rise to an unusually powerful (if not physiologic) immune response, whereas peptides of donor antigens would not be expected to be any more powerful than those of other histocompatibility antigens. If the recent trend toward emphasizing the importance of indirect responses is correct, then new explanations will be required for why allogeneic MHC antigens are more important than other donor antigens in causing graft rejection.

Indirect Presentation and Donor-Specific Tolerance. The availability of indirect recognition also has implications regarding the potential effectiveness of some strategies to achieve donor-specific nonresponsiveness. Many of these strategies seek to present donor antigens to immunocompetent recipients in a manner that leads to T-cell downregulation rather than activation. If these strategies only involve manipulations of donor APCs, they may be ineffective in preventing indirect immune responses because these depend on recipient APCs (246).

Why Does Donor APC Depletion Prolong Graft Survival? Numerous studies have demonstrated that depletion of donor APCs can prolong graft survival (112–116,259). Although this result is in keeping with the central role ascribed to APCs in graft rejection, it is not obvious why donor APC depletion should be effective if the indirect pathway is available to initiate graft rejection. A possible explanation for this observation may be that the donor APCs transfer antigens from the graft to recipient APCs in the draining lymph nodes. Thus, donor APC depletion might prevent both direct and indirect activation at least until APCs of the recipient repopulated the donor graft. An alternative explanation might be that donor APCs are essential for the sensitization of the effector cells responsible for graft rejection, whereas indirect presentation is available for the sensitization of helper cells. Potential effector cells therefore might undergo anergy as a result of encountering donor antigens directly only on parenchymal cells of a graft. In this case, donor APCs also would be required despite the potential importance of the indirect pathway.

Communication Between Indirect Helper Cells and Direct Effector Cells. The possibility that helper cells might be sensitized by recipient APCs while effector cells might be sensitized by donor APCs highlights a special feature of alloreactivity compared with ordinary immunology: that in the case of graft rejection, two different populations of APCs, potentially expressing different MHC antigens, may be involved in the process. This special circumstance creates a unique problem in transplantation immunology: that sensitization of different subpopulations of T cells may occur without the physical linkage afforded by the expression of different determinants on a single APC. The absence of such physical linkage may be important in limiting the availability of help from one subpopulation during the sensitization of another subpopulation. This issue is discussed in more detail below, in the section on the regulation of graft rejection.

Presentation of Donor Antigens by Recipient Class I versus Class II Antigens. Another conceptual problem associated with indirect pathways stems from the general principle in fundamental immunology that peptides of exogenous antigens tend to be presented by MHC class II antigens while those of endogenous antigens are generally presented by MHC class I molecules (260,261). Strictly applied, this principle would imply that indirect recognition would only generate responses to donor peptides presented by self class II molecules, whereas sensitization to donor peptides in association with class I molecules would have to occur by direct presentation. However, numerous exceptions to this principle have been reported, and there is good evidence that peptides of exogenous allogeneic antigens can be presented by recipient class I molecules (262–264).

Bevan demonstrated that indirect sensitization of $CD8^+$ cells can occur in vivo when he described the phenomenon of cross-priming (75). He showed that when minor antigen-disparate grafts with MHC antigens of type A were placed on MHC (A × B) F1 recipients, the $CD8^+$ cells of these recipients became sensitized to the minor antigens presented by both A and B types of class I MHC molecules. Despite these findings, it is still far more common to talk about indirect responses in terms of $CD4^+$ T cells than in terms of $CD8^+$ sensitization by modified self class I molecules. As a mechanism for sensitization of helper cells, indirect $CD8^+$ sensitization is unlikely to be important because the help generated by $CD8^+$ cells has been found to be useful only for the $CD8^+$ cells themselves. Because $CD8^-$ effectors, sensitized indirectly, would not generally recognize determinants expressed in the graft, the $CD8^+$ helper response, which might generate such $CD8^+$ effectors, has generally been thought to be irrelevant for purposes of graft rejection. On the other hand, there is increasing interest in the possibility that indirect effector mechanisms may contribute to graft rejection. In addition, even if the effector mechanisms are unimportant, the cytokines produced by $CD8^+$ cells sensitized indirectly may contribute to the overall regulation of the immune response (265). Thus, the question of how $CD8^+$ indirect sensitization might affect graft rejection remains one of the open issues in transplantation immunology.

Indirect Effector Mechanisms. The classical model of rejection in Fig. 8 suggested that the effector population was sensitized directly just as the helper population was. However, with recognition that helper sensitization could occur through the indirect pathway, it was natural to consider the possibility that effector populations also could be sensitized indirectly, or that inflammatory processes stimulated by indirect helper T cells might cause graft rejection without the participation of any effector T cells that recognize donor antigens directly. These types of rejection mechanisms have been referred to as indirect effector mechanisms and are considered more fully in the next section.

Pathways of Alloreactivity: Effector Mechanisms

Although there are many controversial aspects of helper T-cell function, there is even more uncertainty about the effector mechanisms of cell-mediated graft rejection. Originally, the delayed-type hypersensitivity (DTH) response was the only *in vivo* mechanism known for cell-mediated tissue destruction; thus, for many years transplantation immunologists assumed that it was responsible for graft rejection. Subsequently, the cytotoxic function of T cells was identified, with the attractive feature that a mechanism involving these cells could account for the precise selectivity of graft destruction. Therefore, many transplantation immunologists began to assume that cytotoxic T cells were the important effector cells, and this was the assumption incorporated in the model shown in Fig. 8. As with the helper pathways, recent evidence has suggested that the depiction of effector function in Fig. 8 is too simple. An especially troublesome issue is to determine whether cell-mediated effector mechanisms necessarily require T cells that are specific for antigens expressed by the donor tissue or whether an indirect effector mechanism initiated by T cells specific for modified self MHC antigens also might be sufficient. The following discussion reviews the important data available on these issues and suggests that the almost conflicting conclusions drawn from different types of experiments make it likely that more than one effector mechanism is available, depending on the circumstances.

In Vitro *Studies of Effector Mechanisms*

In vitro studies have been used to examine T cell effector functions in transplantation immunology just as they have for the analysis of helper function. A standard assay measuring an alloreactive effector function is the cytotoxic T-lymphocyte (CTL) or cell-mediated lympholysis (CML) assay measuring T cell–mediated cytotoxicity against allogeneic targets. Alloreactive CTLs can easily be generated from naive T cells after about 5 to 7 days of *in vitro* stimulation with MHC-disparate cells. Generation of CTLs to peptides of minor antigens presented by self MHC molecules, however, requires that the T cells first be primed *in vivo*. The amount of cytotoxicity measured *in vitro* is a function of both the helper activation and the number of precursor CTLs available at the start of the *in vitro* culture. Therefore, to focus on just the cytotoxic effector function, the assay is often performed with the addition of exogenous helper factors, such as IL-2, in order to provide an excess of help. The assay also can be quantified by measuring precursor frequencies of cytotoxic T cells using limiting dilution cultures.

Because alloreactive T cells to foreign MHC antigens can be measured even in naive animals, the standard CML assay is inadequate to determine whether CTLs have been primed in recipients that have rejected MHC-disparate grafts. Therefore, efforts have been made to modify the assay to measure the effect of *in vivo* events. For example, peritoneal T cells from mice that have recently rejected grafts can kill donor targets even without the period of *in vitro* sensitization. In addition, determination of the precursor frequency of alloreactive T cells, measured under modified conditions, can sometimes differentiate recently activated from naive cytotoxic T cells (266). In general, however, it has been difficult to

measure reliably the effect of *in vivo* CTL priming to MHC alloantigens. On the other hand, generation of cytotoxic T cells to minor histocompatibility antigens does require *in vivo* priming and therefore reflects *in vivo* sensitization.

The *in vitro* CML assay has been used to determine the pathways of alloreactive T-cell cytotoxic function. The results of these assays are summarized in Table 5. $CD8^-$ cytotoxic T cells reactive with donor class I antigens are the most frequent effectors *in vitro*, and $CD8^+$ cytotoxic cells specific for self class I antigens modified by allogeneic peptides also can be detected after *in vivo* priming (267–269). In addition, $CD8^-$ cytotoxic cells specific for allogeneic class II antigens can be detected, although they would not be predicted in classical immunology (206). $CD4^+$ cytotoxic cells, specific for allogeneic class II antigens, also can be measured *in vitro* (211). Thus, as for helper cells, there are multiple pathways for generating alloreactive cytotoxic T cells *in vitro*.

In Vivo *Analysis of the Cellular Effector Mechanism*

The Selectivity of Allograft Rejection. A critical feature when considering in vivo effector mechanisms of graft rejection is the selectivity by which the process destroys foreign but not self tissues. Experiments illustrating this selectivity have been performed by placing syngeneic skin grafts adjacent to allogeneic grafts on a single bed. The inflammation of rejection shows a perfect demarcation at the division between the two grafts (270,271). An even more dramatic demonstration of this selectivity has been achieved using skin grafts from tetraparental (allophenic) donors, animals produced by fusing embryonic cells of two parental pairs. Grafts from these animals represent a mosaic of two kinds of cells, interspersed throughout the tissue, each cell bearing one of the two sets of parental MHC antigens. When these grafts are placed on animals that are syngeneic with one of the sets of MHC antigens, there is at first a generalized inflammatory process that appears to destroy the entire epithelium, but some syngeneic tissue remains after the rejection process subsides and all the allogeneic cells have been destroyed (272–274). Thus even when different cell types exist side-by-side, the process of graft rejection shows selectivity.

Experiments such as these often have been taken to imply that graft rejection must take place by the cell-by-cell destruction of allogeneic tissue. This is not the only interpretation, however, because the syngeneic elements that eventually survived from the tetraparental grafts might actually represent the product of epithelial seeding by a few syngeneic cells that remained as the tissue around them died. Furthermore, the experiments with the allophenic skin grafts did reveal a nonspecific inflammatory process early in the rejection process (274). Other types of experiments mixing donor and recipient cell types in a single skin graft also have been performed, using skin from bone marrow chimeras in which the APCs of the skin have been replaced by cells derived from the donor bone marrow (275). When transplanted to syngeneic recipients, skin grafts from these chimeras provide a graft in which only the APCs express alloantigens. The results of these experiments showed that entire skin grafts can be rejected when only the APCs are foreign, although this rejection is more apt to occur when there are large antigenic disparities and when the recipient has been previously sensitized to the foreign antigens. Thus, nonselective destruction of grafted tissue can occur, especially if the inflammatory response is sufficiently vigorous.

Analysis of the Cells Invading Allografts and the Immunopathology of Rejection. Another approach to study the effector mechanisms of graft rejection has been to identify the actual cells that invade a graft by immunohistochemical staining. Such studies, using sponge matrix allografts and rejecting allogeneic organs, have shown that many types of cells are present during graft rejection, including $CD4^+$ and $CD8^+$ T cells, NK cells, and macrophages (276–284). There are relatively few B cells, however, and some T-cell subpopulations (including L-selectin cells) are largely excluded from the graft (285,286). Therefore, some selectivity must exist in the process that recruits the invading cell populations.

The number of invading T cells in a graft is not necessarily related to the speed of the resulting rejection. Whole MHC and class II–disparate grafts generally elicit dense cellular infiltrates, whereas class I–disparate grafts are generally sparsely infiltrated. Indeed, the density of the cellular infiltrate in the case of a class I only difference is not significantly different from the density in a syngeneic graft (285), and the number of invading cells sometimes appears insufficient to mediate rejection by cell-to-cell contact with every allogeneic target. This finding has suggested that certain critical elements of the graft, such as its blood vessels, are the actual site of graft destruction and, indeed, endothelialitis is an important hallmark of clinically significant rejection activity (287). The number of cells within minor antigen–disparate grafts is generally far greater than the number invading grafts with class I only differences, even when the rejection of the class I grafts is faster (285). In addition to the implication that the degree of cellular infiltrate may not correlate with the strength of active rejection, these findings also suggest the possibility that only a small portion of the T cells within a graft may actually have specificity for the allogeneic antigens. The role, if any, of the other invading lymphocytes in rejection is unclear.

Further analysis of the invading cells within rejecting allografts has been undertaken by in vitro propagation of the T cells derived from rejecting organs (283,288–295). Most reports of such efforts have indicated that these T cells are polyclonal and that both cytotoxic and IL-2–producing lymphocytes of both $CD4^+$ and $CD8^+$ lineages can be obtained (296,297). A few reports have suggested that an oligoclonal T-cell response occurs during allograft rejection (298). Overall, however, because so many cell types with so many functions have been identified in rejecting allografts, this type of phenotypic and functional analysis of the invading cells has not been helpful in identifying the effector mechanisms of graft rejection.

In addition to phenotypic and functional analyses of invading T cells, interesting studies recently have been performed correlating the onset of graft rejection with the ability to detect immunologically active proteins in the invading lymphocytes (299–307). For example, PCR has revealed high levels of message for IL-5 in liver grafts showing clinical evidence of rejection, although the role of this cytokine in the rejection mechanism is not clear (308). In addition, graft rejection can be correlated with the presence of perforin, granzymes, and proteases associated with cell-mediated cytotoxicity (309–316).

Studies of Effector Mechanisms Using Knock-Out Mice. Genetically engineered mice lacking the expression of particular genes have been used to study the mechanisms of graft destruction in at least two ways. First, mice lacking the expression of some of their MHC antigens have been used as donors to determine whether grafts can be rejected when they fail to express the determinant rec-

ognized by the effector T cells. For example, skin grafts from class II knock-out mice have been placed on SCID or nude recipients reconstituted with $CD4^+$ but not $CD8^+$ T cells. These experiments have shown that the class II–deficient grafts can be rejected by $CD4^+$ cells alone, but much more slowly than when $CD8^+$ cells are also present (224). Thus, an indirect effector mechanism mediated by $CD4^+$ cells in this case appears to be much less effective than one involving direct recognition of donor antigens. The ultimate experiment to test the effectiveness of an indirect effector mechanism would be to use completely MHC-deficient donors (317). Experiments of this type have been performed using the mice generated by crossing class II and β2m knock-out animals (318). Unfortunately, the results of these experiments have indicated that there is sufficient residual class I antigen expression by the double knock-out mice that rejection of skin grafts can still be mediated by $CD8^+$ effector cells specific for the class I antigens, making the use of skin grafts from these donors ineffective for answering the experimental question (227,319). On the other hand, the rejection of other types of tissues, such as pancreatic islets, is diminished substantially by the reduced expression of donor MHC antigens unless the MHC-deficient islets are placed in xenogeneic recipients (320–324). Thus, these results again suggest that an indirect effector mechanism is a relatively ineffective way of destroying transplanted tissue, unless the inflammation generated by the indirect response is especially powerful.

The other way that genetically engineered mice have been used to examine rejection mechanisms has been to use mice lacking the expression of particular genes as recipients of transplants. A striking result of these types of experiments is that rejection of all types of organ transplants can occur in the absence of the key components of T-cell cytotoxicity such as perforin and Fas ligand (325,326). Thus, despite the strong correlation between expression of perforin and evidence of clinical rejection described above, these experiments strongly suggest that cytotoxicity is not essential for graft rejection. Cytotoxicity by T cells may represent one of several mechanisms for graft destruction, or the presence of cytotoxic T cells in rejecting allografts may simply be a marker for the sensitization of effector T cells that actually utilize a different mechanism. The use of other types of knock-out mice as recipients lacking various cytokines also has failed to reveal any single molecule that is essential for the mechanism of graft destruction (327–330).

Correlation Between In Vitro *and* In Vivo *Results*

Another approach to analyzing effector mechanisms has been to examine the correlation between graft rejection *in vivo* and the ability to measure *in vitro* cell-mediated cytotoxicity. A strong correlation would support a T cell–mediated cytotoxic mechanism, whereas a breakdown in this correlation would suggest alternative mechanisms.

One way to measure this correlation is to deplete selected T cells *in vivo* before placing grafts with limited antigenic disparities. For example, such studies have shown that $CD4^+$ cells alone but not $CD8^+$ cells alone can reject skin grafts with only class II antigen disparities (41,146,147,221). This result correlates with the *in vitro* experiments showing that both $CD4^+$ and $CD8^+$ cells contain precursors of cytotoxic cells specific for allogeneic class II antigens, but that only the $CD4^+$ population has a helper pathway to generate mature class II–specific CTLs. The outcome of many other experiments of this sort correlate with the *in vitro* results for CTL generation.

The most controversial experiments of this type have involved studies of rejection of class I–only disparate grafts by recipients depleted of $CD8^+$ T cells (41,146,147,214,220,221,331). Graft rejection frequently has been demonstrated in this situation even though *in vitro* assays have generally failed to reveal cytotoxic $CD4^+$ cells specific for class I alloantigens. Thus, these results appear to violate the correlation between graft rejection and the ability to measure *in vitro* cytotoxicity. Rosenberg and Singer, however, demonstrated that mice depleted of $CD8^+$ T cells by antibody treatment still have a population of cytotoxic precursors (apparently of the $CD8^+$ lineage despite the absence of the CD8 antigen) that require *in vivo* priming and help from $CD4^+$ T cells for their activation. These investigators have demonstrated the presence of $CD4^+$, $CD8^+$, $\alpha\beta^+$ cytotoxic T cells after graft rejection in the mice that were treated with anti-CD8 antibodies (222,332). These results suggest that depletion of $CD8^+$ cells *in vivo* may not always eliminate all cytotoxic cells of this lineage and that the rejection of class I–disparate skin grafts apparently by $CD4^+$ cells alone is not actually a violation of the correlation between *in vitro* CTL activity and *in vivo* graft rejection. Other investigators have disputed Rosenberg and Singer's conclusions, suggesting that they apply only to the limited antigenic disparities generated by the comparison of the class I mutant $H\text{-}2^{bm}$ mice with wild-type H-2b mice. These other investigators suggest that the larger number of foreign peptides generated by more disparate class I antigens are sufficient to generate an effector mechanism mediated by $CD4^+$ cells specific for class I peptides presented by class II molecules (333). These results do not distinguish a direct from an indirect effector mechanism because the class II molecules of the donor and recipient are identical in these experiments, but they do suggest a lack of correlation between *in vivo* rejection and *in vitro* cytotoxicity because $CD4^+$ cytotoxic T cells have not been detected after rejection in these experiments.

Another important challenge to the hypothesis that cytotoxic T cells cause graft rejection comes from examination of *in vitro* cytotoxicity after rejection of grafts that differ only in minor histocompatibility antigens. Because CTL activity to minor antigens can only be measured *in vitro* after *in vivo* priming, minor disparate graft rejection provides a good opportunity to test whether every case of rejection is associated with the development of CTL activity. A particularly good model to test this correlation is the rejection of murine skin grafts, which differ by only the H-Y antigen because some strains, but not others, can reject skin grafts with only this single minor antigen disparity. Experiments from Simpson's laboratory have indicated that the rejection of H-Y grafts is not always associated with measurable *in vitro* cytotoxicity, whereas in other cases the development of CTLs *in vitro* occurs despite the absence of graft rejection (334–336). Although these results obviously challenge the role of CTLs in graft rejection, they too are controversial.

Uncertainties Regarding the Effector Mechanisms of Graft Rejection

All of the approaches described above have failed to provide clear evidence demonstrating a single effector mechanism for graft destruction. Although the results do suggest that cytotoxic T cells are not the only effector cells involved, they do not demonstrate what other effector mechanisms are involved and they fail to answer the basic question of whether effector T cells must neces-

sarily recognize donor MHC antigens directly in order to achieve the selectivity of graft destruction. As diagrammed in Fig. 10, the evidence is consistent with the complex view that multiple effector mechanisms exist, some involving cytotoxicity and some not, and some with specificity for donor antigens, and others involving an indirect effector process (221,281,337–346). The evidence at this point does suggest that indirect effector mechanisms are less efficient than ones involving direct recognition unless the stimulus to a nonspecific inflammatory response is especially powerful. All of these conclusions are tenuous at this time, however.

Potential Final Mediators of T Cell–Dependent Effector Mechanisms

Whether or not there are indirect effector mechanisms for graft destruction, the final mediators of cell-mediated rejection may not be T cells themselves, but rather other components of the immune system that depend on helper T cells. There are several candidates for such mediators of graft destruction. Classical DTH responses are thought to depend on the activation of macrophages by helper T cells through production of IFN-γ. In turn, the destruction of tissues by activated macrophages often may involve the production of toxic molecules such as nitric oxide (347). Although an effector mechanism involving macrophages would appear to lack selectivity, the process might still cause limited tissue destruction if the donor cells (such as pancreatic islets) are especially sensitive to these inflammatory mediators, or if donor blood vessels in the immediate vicinity of the activated cells are especially likely to be injured by the inflammatory response. Cytokines are clearly involved in the mechanisms of graft rejection. However, most of the obvious examples of their participation involve their role in the helper mechanisms of T-cell sensitization. It is likely, however, that some cytokines, such as TNF-α, may themselves be toxic to allogeneic tissues.

Chronic Rejection (B and/or T Cell-Mediated)

Most experimental studies of rejection are performed without immunosuppression. Therefore, graft destruction usually occurs within the first several weeks by one of the mechanisms described above. In clinical practice, however, the use of immunosuppression usually allows graft survival for much longer periods of time. Nonetheless, clinical survival statistics show that even when 1-year graft survival has been achieved, the loss of transplanted organs continues to occur at a rate of about 3% to 5% per year, and a significant portion of this loss appears to be due to immunologic mechanisms. The term "chronic rejection" has been used to describe this late process of graft destruction. Because immunosuppressive reagents have become more effective at controlling acute rejection, chronic rejection has emerged as one of the most important problems in clinical practice. Indeed, Fig. 11 shows that although there has been ongoing improvement over the past 30 years in the 1-year graft survival rates for kidney transplants, the half-life for organs that have survived for 1 year has not changed significantly over that entire period of time (348). As a result of this ongoing loss, only about 50% of transplants are still functioning 10 years later.

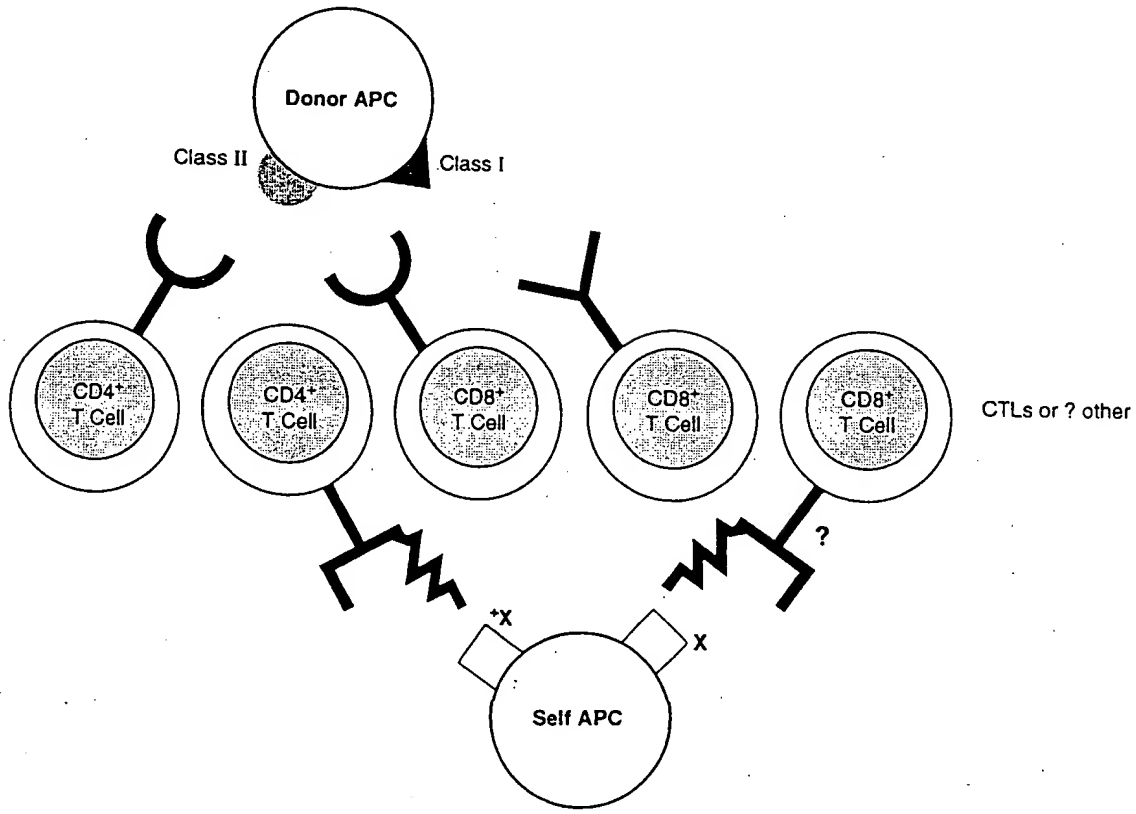


FIG. 10. Complex mode of T-cell effector pathways.

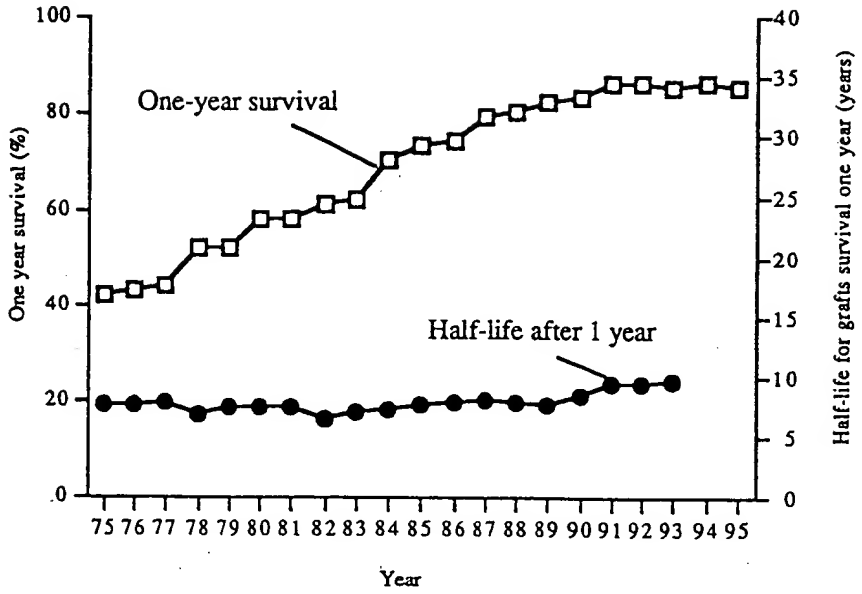


FIG. 11. One-year graft survival and chronic half-life over time for kidney transplants.

Although almost every type of organ transplant suffers from chronic rejection, the pathologic manifestations are different in each case. Kidney biopsies tend to show interstitial fibrosis along with arterial narrowing from hyalinization of the vessels. In the heart, the process is manifested principally as a diffuse myointimal hyperplasia proceeding to fibrosis of the coronary arteries that has often been referred to as accelerated atherosclerosis. Chronic rejection in lung transplants primarily affects the bronchioles with progressive narrowing of these structures and is referred to as bronchiolitis obliterans. The liver may be the one type of organ transplant that is relatively resistant to chronic rejection, but the progressive destruction of bile ducts referred to as the vanishing bile duct syndrome may be another manifestation of this process.

Some of the causes of chronic graft destruction may not be immunologic in origin (349,350). Potential factors that have been considered include the initial ischemic insult, the reduced mass of transplanted tissue (especially in the case of kidney transplants leading to hyperfiltration injury), the denervation of the transplanted organ, the hyperlipidemia and hypertension associated with immunosuppressive drugs, the immunosuppressive drugs themselves, and chronic viral injury. Nonetheless, although these factors undoubtedly contribute to the process, there is a marked difference in survival between syngeneic and allogeneic transplants in experimental models. In addition, native hearts in kidney transplant recipients do not show manifestations of chronic rejection and vice versa. Thus, there is almost certainly an important immunologic component in most cases of chronic rejection.

Several important observations regarding chronic rejection have emerged from clinical practice. First, the process is frequently associated with the presence of antidonor antibodies. This was first recognized in the case of kidney transplants when a high correlation was found between the presence of alloantibodies and the hyalinization of renal arteries on late biopsy specimens (351). The same correlation has been found for other organs as well (352). Second, the process of chronic rejection is usually refractory to increases in immunosuppressive therapy, in contrast to acute rejection episodes, which almost always respond to treatment. Third, there is a high correlation between the onset of chronic rejection and a history of early acute rejection episodes (353). Together these clinical observations have suggested to some that chronic rejection is the result of chronic B-cell alloantibody production. They have suggested to others that chronic rejection requires the early sensitization of the immune system to donor antigens. Both suggestions may be correct, but neither the logic nor the evidence fully supports these conclusions. In the first place, alloantibody production often reflects indirect T-cell sensitization; hence, it might equally well be a marker for other rejection mechanisms as opposed to a cause of chronic rejection. In addition, early rejection episodes probably reflect primarily the degree of antidonor immunoreactivity and may not themselves be required for chronic rejection. Therefore, even if sufficient immunosuppression were given to prevent acute rejection, chronic rejection still might occur when the suppression was reduced to levels tolerable over the long term, even if acute rejection had never occurred. Finally, experimental studies have suggested that the mechanisms of chronic rejection are not absolutely dependent on either antibody formation nor on the occurrence of acute rejection episodes (354,355).

The uncertainties that arise from the interpretation of the clinical data make it important to develop experimental models for studying the mechanisms of chronic rejection. However, it is difficult in the laboratory to mimic a process that may take 5 or 10 years to develop in patients treated with immunosuppressive drugs. Thus, the effort to study chronic rejection experimentally has depended on surrogate short-term pathologic markers that are thought to predict the long-term changes of chronic rejection. In particular, these studies have concentrated on the development of the myointimal proliferation that is thought to be the precursor of the chronic vascular changes typically observed in patients. Both in rodents and in pigs, this has often been done with heart transplants after an initial period of immunosuppression that prevents acute rejection (356). All of these experimental studies are subject to the caveat that the surrogate pathologic lesion occurs much earlier than the typical changes of chronic rejection in clinical patients. Thus, the process being studied experimentally may not be the same as the clinical process.

Pathologic Manifestations of Experimental Chronic Rejection

The typical pathologic features of the experimental lesion associated with chronic rejection are shown in Fig. 12 (356). The marked narrowing of the vascular lumen is caused by the substantial proliferation of endothelial and then smooth muscle cells. Associated with this proliferation is progressive destruction of the media. With further time, the cellular proliferation becomes less pronounced and is replaced by concentric fibrosis that narrows the vascular lumen. Immunohistologic staining indicates that there is increased expression of several adhesion molecules during the early manifestations of this process (354) and easily detectable levels of several cytokines and factors (357–361), including nitric oxide synthase (362), acidic fibroblast growth factor (363), insulin-like growth factor (364), and endothelin (365,366).

Immunologic Mechanisms of Chronic Rejection

With the availability of animal models, it has become possible to examine the immunologic mechanisms responsible for chronic rejection, using the same genetic modifications and manipulations of the recipient that have been useful in the studies of other rejection mechanisms. Studies in pigs have suggested that the vascular changes are more apt to develop when there are class I antigenic disparities than when there are only class II disparities and have suggested that the lesion depends especially on $CD8^+$ T cells (367). Mouse studies, however, have indicated that either $CD4^+$ or $CD8^+$ T cells can produce the lesion and that either class I or class II antigenic disparities are sufficient to stimulate chronic rejection (368). The finding that class II antigenic disparities are themselves sufficient is especially important because in mice these antigens are not expressed on the vascular endothelium, indicating that the lesion can develop even when a target antigen is not expressed on the cell type that shows the most striking proliferation (354). In keeping with the prediction of many clinical studies, adoptive transfer experiments into SCID mice have shown that alloantibodies in the absence of T cells can induce the typical pathologic vascular changes (369). However, T cells without B cells also have been shown to cause the lesion, although there may be somewhat less tendency to progress to end-stage fibrosis (355). Several studies have indicated that the induction of donor-specific tolerance can prevent the development of the vascular changes of chronic rejection, although not all of the short-term manipulations that have been effective in preventing acute rejection have necessarily prevented the later onset of chronic rejection.

From these data, it is difficult to determine a single immunologic mechanism that is the cause of chronic rejection. Probably, the process represents the manifestation of chronic injury from many different types of ongoing immune response and that the particular manifestation of this injury depends on the organ involved. In most cases, the injury is reflected in the vascular changes described above, but in the case of the lung or the liver, chronic injury may cause changes most prominently in the bronchioles or the bile ducts. Because it is assumed that stimulation of direct immune responses is likely to diminish over time as donor APCs are replaced by recipient APCs, it is commonly assumed that the predominant immune response that causes chronic rejection occurs through the indirect pathway. However, there is no evidence at this time to support this assumption.

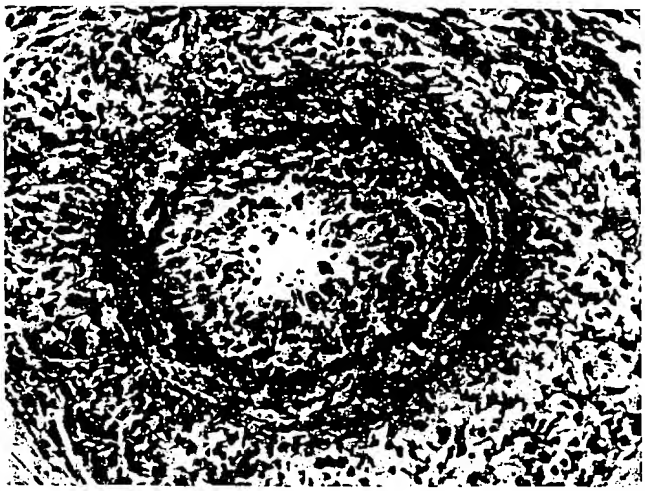


FIG. 12. Moderately advanced (stage 3) arterial lesion in an experimental form of chronic rejection, showing marked cellular expansion of the intima, luminal stenosis, and a prominent adventitial infiltrate that invades the media. B10.A to B10.BR mouse heart transplant on day 56, × 200, elastic tissue stain. Reprinted with permission (354).

PHYSIOLOGIC INTERACTIONS REGULATING GRAFT REJECTION

The preceding sections have described the interactions between donor antigens and the recipient immune system that lead to graft rejection. This section addresses the regulatory elements that control this process. Because the regulation of complement and endothelial activation already have been described in the sections on humoral mechanisms of rejection, this discussion will concentrate on the regulation of T-cell responses. First, we consider the process of T-cell sensitization, then we consider the interactions required between sensitized helper and effector cells, and finally we consider the regulation of effector cell activity.

Regulation of Sensitization

Tissue Damage and Inflammatory Signals

Although it is possible for T-cell sensitization to occur in the presence of resting dendritic cells, many types of APCs require activation before they gain full APC function. For this reason, and also because of changes in adhesion molecule expression and cell trafficking, one of the important elements controlling T-cell sensitization is the release of inflammatory cytokines, such as IFN-γ, with its potent ability to activate macrophages (370). Tissue injury from any source is an important stimulus for releasing such cytokines; thus, the concept that a danger signal helps regulate graft rejection is important in transplantation immunology (371). All forms of transplantation involve ischemic and traumatic injury to the donor tissue, which may be one of the reasons that rejection episodes occur most frequently early after transplantation. In addition, later nonimmunologic inflammation, either occurring in the transplanted organ or perhaps elsewhere in the body, may trigger late rejection episodes. On the other hand, it would be wrong to picture the role of nonspecific danger signals as the dominant feature

controlling graft rejection. For example, skin transplants placed on SCID recipients can be allowed to heal for long periods before immunologic reconstitution of the recipients, but rejection always occurs when this is done. Similarly, even achieving organ transplant survival for many years in clinical practice is rarely sufficient to allow the cessation of immunosuppression. Thus, it is better to picture the antigenic disparity and the recipient's immunoresponsiveness as the dominant features controlling graft rejection, while danger signals may influence the timing, intensity, or character of the immune response.

Costimulatory Signals Involved in T-Cell Activation

The special features of APCs involve not only their ability to present foreign antigens on their surface but also to provide additional signals for T-cell activation. This second component of T-cell activation is often referred to as the "second signal", although it more likely involves several different elements (372–375). The likely components of the second signal include the lymphokines secreted by APCs, including IL-1, and signals transmitted after binding of T-cell accessory molecules with their ligands on APCs. These include the interaction of CD4 or CD8 with monomorphic determinants on MHC antigens, LFA-1 with ICAM-1, 2, or 3, CD2 with LFA-3 (CD48), CD40 with CD40 ligand, CD28 with B7.1 and B7.2, and the function of 4-1BB. In addition, lymphokines secreted by T cells, such as IL-2, may further contribute to the second signal for both themselves and other cell populations (376–378).

An important feature of the second signal is that in the absence of some or all of its components, T cells stimulated through their antigen receptor may be anergized (as discussed below in the section on tolerance induction). Because many of the cells of a transplanted organ are not APCs, the induction of anergy would seem likely to be initiated after every organ transplant, a process that is in competition with the activation events stimulated by APCs. Early after transplantation, this competition probably favors APC activation because allograft rejection is almost universal, but the stimuli may be more evenly balanced months or years after transplantation when the donor APCs have mostly been replaced.

Downregulating Signals Following T-Cell Activation

It has become increasingly apparent in recent years that cell-mediated immune responses are controlled by downregulating interactions, such as by the ligation of CTLA-4 and by the interaction between Fas and Fas ligand (379–381). In transplantation, there is evidence that high levels of expression of Fas ligand on some or all of the cells of donor tissue may prevent rejection (382) and that Fas ligand may be partly responsible for lack of rejection when tissues are transplanted to some privileged sites, such as the testis or the anterior chamber of the eye (382). These findings suggest that a promising approach for preventing rejection might be to manipulate these molecular interactions (381). However, our understanding of these downregulating signals is not adequate at this time to generate effective strategies for this purpose. For example, overexpression of Fas ligand in pancreatic islets has tended to make them more susceptible to destruction rather than to provide them with protection from rejection, and CD28 knock-out mice have been found to reject allografts, despite unopposed signaling through CTLA-4 (383). It is likely, however, that a better understanding of the downregulating events controlling immune responses will provide new approaches for preventing graft rejection in years to come.

Regulatory Cytokines

Many cytokines play a role in the regulation of graft rejection. However, because almost none of the cytokine or cytokine receptor knock-out mice have demonstrated a defective phenotype for graft rejection, it is impossible to describe more precisely which cytokines provide particular regulatory functions. It is usually assumed that IL-2 is important as a helper cytokine for effector T cells, but its role can apparently be performed by other molecules (377). IFN-γ is thought to play a role in activating other cells in the process of graft destruction and in upregulating the expression of antigens, especially on donor vascular endothelium (384). However, graft rejection also occurs rapidly on the IFN-γ knock-out mice (385,386). Several studies with antiinflammatory cytokines, such as IL-10 or transforming growth factor (TGF)-β, suggest that these may modify the process of graft destruction (387–389), whereas proinflammatory cytokines such as TNF-α are thought to enhance the process (361). On the other hand, IL-10 can enhance cytolytic mechanisms of islet graft rejection (390). Studies of tolerance induction have suggested that in some cases shifts in the balance of Th1 and Th2 cytokines may be important in determining graft rejection versus acceptance (391). However, the difference in outcome based on Th1 versus Th2 production is far from clear, as will be discussed further below (392–397). Thus, although cytokines are undoubtedly of enormous importance in causing and regulating the processes of graft rejection, our understanding of their role is poor at this time.

The Presence of the Transplanted Organ

Although early rejection episodes occur with most types of organ transplants, there are some exceptions to this rule. Kidney and liver transplants in mice can survive for long periods without immunosuppression even with MHC disparities (398–400), and there have been cases of prolonged liver transplant survival in pigs without immunosuppression (401). In addition, many types of rodent transplants, such as mouse heart transplants, require only a short course of immunosuppression to achieve prolonged survival (402,403). Furthermore, in the experimental animal studies, the long survival of these transplanted organs often diminishes, or even prevents, the subsequent rejection of antigenically identical skin grafts that would have been rejected rapidly by naive animals (398,404). Even in clinical transplantation it appears that the long survival of a transplanted organ may diminish the rejection response because much less immunosuppression is required late after transplantation than in the early period. Thus, there is substantial evidence that the mere survival of a transplanted organ generates a powerful regulating force that inhibits the specific anti-donor immune response.

As discussed below, the induction of anergy, as donor grafts lose their APCs, is one possible mechanism by which this might occur. However, it is also possible that downregulating signals from the allograft, even potentially from allogeneic APCs, or that changes in cytokine production contribute to the inhibition of graft rejection caused by the persistent survival of the organ.

There are two important features to emphasize regarding the capacity of transplanted organs to regulate their own survival. First,

their capacity to do so often confuses the results of experimental studies designed to test tolerance-inducing strategies. For example, it is frequently reported that a particular form of immunosuppression induces tolerance when provided at the time of murine cardiac transplantation. Although the result may be accurate, the conclusion that the form of immunosuppression used leads to tolerance is not justified. The long survival of the transplanted heart, rather than the immunosuppression that achieved it, may be responsible for the tolerant condition. This issue can be tested by removing the transplanted organ to see if tolerance persists, or by testing the particular form of immunosuppression with other types of antigenic challenge from the donor. Second, it is important to understand that the processes that downregulate graft rejection as a result of long-term graft survival may be inhibited by the standard forms of immunosuppression that are used clinically to achieve excellent graft survival (405). This is probably because many of the standard immunosuppressive drugs inhibit T-cell signaling and therefore inhibit active processes of tolerance induction. In other words, if T cells never learn that they have encountered donor antigens, they may not generate donor-specific mechanisms that inhibit graft rejection.

Communication Between Helper and Effector Cells

APCs play a role in regulating immune responses by serving as the focus for the interaction between helper and effector cells.

A Three-Cell Model of Helper and Effector Cell Interactions

An important tenet of fundamental immunology is that the cell–cell interactions that generate an immune response generally require intimate contact between the individual cells involved. Mitchison demonstrated this principle in studies of the T–B collaborations leading to antibody production. He showed that T cells, B cells, and the APCs that stimulate them must join together in a three-cell cluster to achieve effective collaboration between the helper T cells and effector B cells (406). Findings such as these have led to the concept that the lymphokines involved in helper function tend to function like neurotransmitters, working only between two closely spaced cells, rather than as hormones acting over large distances (407–409).

In addition to Mitchison, others have performed experiments suggesting that the three-cell cluster model also applies to the interactions between helper T cells, effector T cells, and APCs involved in graft rejection (146,215,410,411). For example, tail skin grafts from class I mutant mice (bm7) placed on B6 recipients are not rejected, apparently because of a lack of helper stimulation. On the other hand, grafts from (bm12 × bm7) F1 mice, which express an additional class II antigen disparity, are rejected. A bm12 graft on one side of a B6 mouse, although itself rejected, does not induce the rejection of a bm7 graft on the other side of the same animal, whereas a (bm12 × bm7) F1 graft on one side of a recipient does induce rejection of a bm7 graft on the other side. These results suggest that the helper factors elicited during rejection of a bm12 graft cannot function elsewhere in the body to assist potential effector cells specific for the bm7 graft. On the other hand, when both the bm12 and bm7 antigens are expressed on the same graft, and therefore on the same APCs, effector cells are generated that can function elsewhere in the body. As diagrammed in Fig. 13, it appears that the helper cells, effector cells, and stimulating APCs must join together in a three-cell cluster to allow efficient helper function for graft rejection (150,412). An attractive feature of the three-cell model is that it provides a mechanism for regulating the availability of help. Responses occurring elsewhere in the body, perhaps stimulated by environmental pathogens, will not generally initiate an immune response to the donor graft.

T-Cell Help for B-Cell Alloantibody Production

T-cell help for B-cell alloantibody production is an example in which several cell populations need to interact to achieve an immune response. Although it is commonly assumed that the B-cell production of antibodies to protein (usually MHC) antigens involves first the production of IgM antibodies, in a T cell–independent process, and later the conversion to IgG antibodies, requiring T-cell help, studies of alloantibody production, at least after skin graft rejection, have actually suggested that even the initial IgM response also depends on $CD4^-$ T cells (149). However, there are two potential pathways by which $CD4^+$ T cells might provide help for alloreactive B cells (413). First, as diagrammed in Fig. 14 on page 1216, recipient $CD4^+$ helper cells might recognize donor class II antigens directly, whereas recipient B cells recognize donor class I MHC antigens. Alternatively, recipient $CD4^+$ cells might recognize donor peptides presented by recipient APCs through the indirect pathway and then provide help to recipient B cells that recognize donor antigens directly. In the first case, the T and B cells would be in close physical association, but in the second case the T cells would interact with the B cells even more intimately, through their recognition of the B cell's class II antigens presenting donor peptides. Experiments to examine these two possibilities have been performed using class II knock-out mice as either donors or recipients and then testing alloantibody production (414). The results have indicated that there are two levels of help that can be provided by $CD4^+$ T cells for B cells. First, the help provided by T cells brought into physical association with B cells through the direct pathway allows B-cell IgM production. Second, the help provided by T cells sensitized indirectly allows B cells to produce IgM antibodies and to convert from IgM to IgG production. In addition to indicating the importance of the indirect pathway in this form of T-cell helper sensitization, these results also suggest that the conversion to IgG alloantibody production can be used as a marker to demonstrate that indirect sensitization of $CD4^+$ T cells has occurred (415–417).

Can a Four-Cell Cluster Activate Effector T Cells?

It is easy to picture how donor APCs, stimulating T-cell responses through direct presentation, provide the focus for a three-cell interaction during T cell–mediated graft rejection because donor APCs can express both the helper determinants and the effector determinants necessary to bring the two T-cell populations together. On the other hand, when the indirect pathway for helper sensitization is considered, recipient APCs will not necessarily express donor MHC antigens and therefore will not generally express the same effector determinants that are present in the graft. Therefore, if an indirect helper response is to generate effector cells that recognize donor antigens directly, $CD4^+$ cells stimulated by recipient APCs would have to provide help for $CD8^+$ cells that would be sensitized by donor APCs. The three-cell model would not predict that productive helper–effector communication would occur under these circumstances. Nonetheless, although the evidence is clear that APCs from one graft and APCs from a second

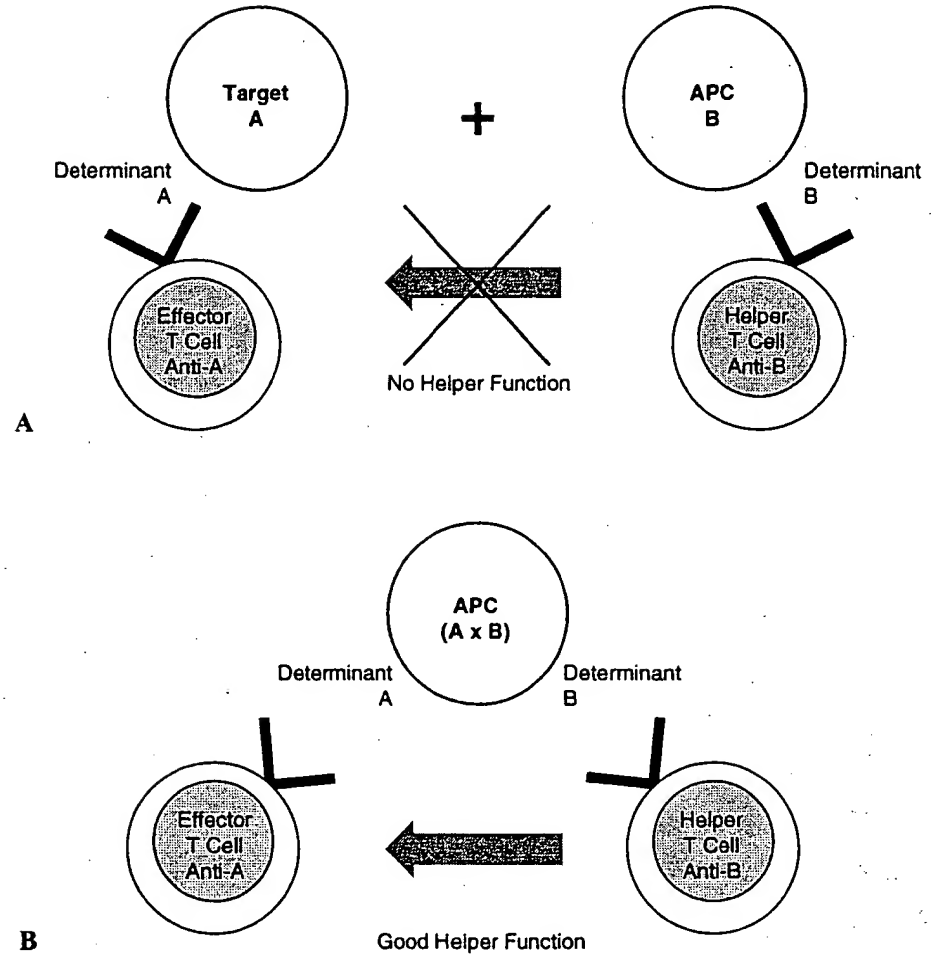


FIG. 13. The three-cell cluster model of sensitization. **A:** Two populations of APCs *in vivo* cannot stimulate help for each other's determinants. **B:** One population of APCs can stimulated help for the different determinants expressed by that population.

graft cannot work together, there is evidence that APCs from a graft and APCs from the recipient can join in a four-cell cluster with helper and effector cells to initiate graft rejection (227,418).

Effector Cell Regulation

The third level of regulatory interactions in graft rejection involves the effector cells after they have been sensitized by contact with donor antigens and been augmented by activated helper cells. Once activated, the effector cells appear capable of functioning anywhere in a recipient where they encounter donor antigens, in contrast to the limited range of helper function (112,419–422). Nonetheless, the function of effector T cells is controlled by regulation of the trafficking of the effector cells and by the accessory molecules involved in the interaction of effector cells with their target cells.

Adhesion Molecules Regulate Effector Cell Trafficking

The regulation of lymphoid cell trafficking by adhesion molecules is one of the expanding areas of fundamental immunology (see Chapter 14). A key feature of this regulation is that naive T cells are kept within lymphatic tissues and that only activated or memory T cells are allowed to circulate into peripheral tissues. This pattern is controlled through the expression of L-selectin and other adhesion molecules expressed by naive T cells that keep them in the lymphatic circulation (423). Activated cells lose this expression and are free to circulate more widely (138).

In addition to allowing activated cells out of lymphatic tissue, adhesion molecules control the entry of cells into foreign tissues. Inflammation alters the trafficking patterns of lymphoid cells through the expression of ICAM-1, ELAM, VCAM-1, and perhaps other adhesion molecules expressed on vascular endothelium (424–427). These molecules bind lymphoid cells, polymorphonuclear lymphocytes, and macrophages to sites of inflammation by halting their passage within vessels and stimulating the transmigration of these cells across the vascular endothelium. The expression of these adhesion molecules changes over time in response to various cytokines and other factors (308,424,425,428).

The cellular infiltrate associated with graft rejection is a special case of inflammation, and recent studies have investigated the unique features associated with allogeneic compared with syngeneic grafts (429). Both types of grafts show a cellular infiltrate during the first

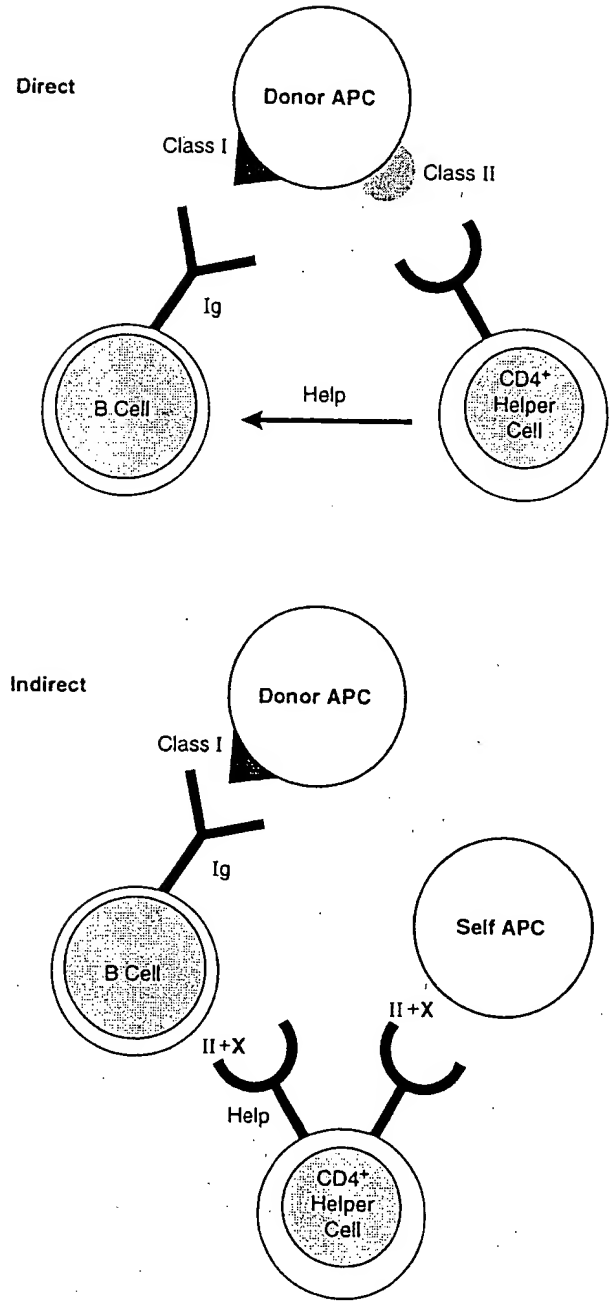


FIG. 14. T-cell help for B-cell alloantibody production.

several days following transplantation, and both types express ICAMs and ELAMs. By about the fourth day after transplantation, however, allografts can be distinguished from syngeneic grafts by the expression of VCAM-1 and by the appearance of IL-2, IL-4, and IFN-γ (430). Further studies of this type may help to elucidate the regulatory elements of effector cell function in graft rejection.

Target Cell Accessory Molecules and Effector Cell Function

In addition to altering cell trafficking, cell surface molecules are important in controlling the interaction of effector T cells with their targets. The T-cell antigen receptor and CD3 proteins are naturally critical in this interaction. The CD4 and CD8 coreceptors, which bind to monomorphic determinants on MHC antigens, also are important (431), as is the interaction of ICAM-1 with LFA-1 (432). Of course these same molecules are also involved in the early stages of T-cell sensitization, but their additional role at the effector stage suggests that interruption of these interactions may alter graft rejection even after T cells have been activated (433,434).

MANIPULATIONS TO PREVENT GRAFT REJECTION

The importance of transplantation immunology lies ultimately in the application of its principles to clinical transplantation. Thus, the critical issue is to determine how the components and regulatory interactions involved in graft rejection might be manipulated to allow graft acceptance. One level of immunosuppression involves nonspecific approaches, reducing the overall immunocompetence of the recipient to all foreign antigens, and the second level seeks to prevent responses only to the antigens of a particular donor. Ultimately the goal is to achieve tolerance, which is lasting donor-specific nonresponsiveness.

Nonspecific Techniques

Standard Drugs

It is beyond the scope of this chapter to review the pharmacology of the nonspecific immunosuppressive drugs commonly used in clinical transplantation. It is important to acknowledge, however, that the major advances in clinical transplantation have been made possible largely by such agents. Most recipients of allogeneic organs receive exogenous immunosuppression in the form of steroids, azathioprine, mycophenolate, cyclosporine, or FK-506. In general terms, both standard and experimental immunosuppressive drugs suppress immune responses by inhibiting lymphocyte gene transcription (e.g., cyclosporine, FK-506), cytokine signal transduction (rapamycin, leflunomide), nucleotide synthesis (e.g., azathioprine, mycophenolate mofetil), or differentiation (15-deoxyspergualin).

Corticosteroids have pleiotropic effects, including inhibition of T-cell proliferation and cytokine gene transcription (435). Azathioprine is an analog of 6-mercaptopurine that acts by inhibiting purine metabolism so as to block cell division. Mycophenolate mofetil is a prodrug of the active metabolite mycophenolic acid, which is related to azathioprine in its inhibition of purine synthesis. Its effect is limited, however, to the enzymatic pathways involved in lymphocyte proliferation, theoretically allowing normal development of other hematopoietic elements. However, significant side effects, including leukopenia, were observed in double-blind randomized trials comparing mycophenolate to azathioprine in renal allograft recipients. A significant reduction in rejection episodes, without any overall effect on early graft survival, was observed in the mycophenolate groups in these trials (436,437). Cyclosporine blocks the generation of IL-2 by T cells and thereby prevents sensitization (438–440). The discovery of cyclosporine played a major role in making cardiac and liver allotransplantation feasible, and it has become a mainstay of many immunosuppressive regimens. The complex of cyclosporine and its cytoplasmic receptor cyclophilin binds to and blocks the phos-

phatase activity of calcineurin, which is an intracellular signaling protein that is essential for transcriptional activation of the IL-2 gene. FK-506 is a macrolide that binds to a member of the intracellular FK-506 binding protein (FKBP) family of intracellular receptors. The FK-506–FKBP12 complex binds to and inactivates calcineurin, and thus has effects quite similar to those of cyclosporine (441).

In addition to the standard drugs for clinical transplantation, several other drugs are sometimes used. Cyclophosphamide is roughly equivalent to azathioprine in its effects at the doses used in transplantation. It is sometimes substituted for azathioprine to avoid particular side effects or with the hope of controlling B-cell responses. Prostaglandin E1 has been found to be immunosuppressive in some experimental models, and it may reduce some of the toxicities associated with other agents. Actinomycin D inhibits bone marrow function and is used occasionally.

Experimental Drugs

The list of standard drugs will almost certainly be modified in the near future by the addition of drugs that are now considered experimental. Rapamycin is a macrocyclic triene antibiotic somewhat analogous to FK-506 (442). Despite binding to the same intracellular binding protein (FKBP12) as FK506, the FKBP12–rapamycin complex does not block calcineurin activity. Instead, rapamycin has a different ultimate target protein known as the mammalian target of rapamycin, and it inhibits T-cell proliferation by blocking signal transduction mediated by IL-2 and other cytokines, not by inhibiting IL-2 production (442). Rapamycin has proved to be a potent immunosuppressive agent in animal studies, although it is not without significant toxicity (442). It is currently being evaluated in clinical trials in combination with other immunosuppressive agents. 15-Deoxyspergualin is a distinctly different agent that has no effect on IL-2 production or utilization (443). It appears to prevent activated T and B cells from differentiating into mature effector cells. It is currently under clinical evaluation for rejection crises and for prophylaxis in highly sensitized patients. Leflunomide is an orally bioavailable prodrug that is converted to the active metabolite A77 1726, which has shown promise in treating acute and chronic rejection in animal models. It prevents lymphocyte proliferation both by inhibiting *de novo* pyrimidine synthesis and by inhibiting the activity of tyrosine kinases associated with cytokine receptors. Leflunomide also can prevent smooth muscle proliferation, and hence may be beneficial in preventing graft vasculopathy (435). Leflunomide has not only prolonged allo- and xenograft survival but also has prevented the production of antidonor antibodies in animal models (444,445).

Anti–T Cell Antibodies

Another form of nonspecific immunosuppressive therapy used both clinically and experimentally is that achieved with antibodies specific for T cells of the recipient. Originally anti–T cell antibodies were obtained from heterologous antisera prepared against lymphocytes or thymocytes of the recipient species (ATG or ALS) (446,447). These powerful immunosuppressants are still used in some induction regimens and for the treatment of rejection episodes. Their major side effects include serum sickness and infectious complications. More recently, monoclonal antibodies (mAbs) such as OKT3, a mouse antibody directed against the CD3 antigen of humans, has become widely used in clinical transplantation (148). Like polyclonal sera, OKT3 is highly efficacious in reversing rejection episodes and is also used in many centers in the first week or two posttransplant to prevent rejection episodes. Other pan–T cell antibodies have been used in clinical trials, including CAMPATH-1, T-12, CBL1, and BTI-322, but none has yet proven more effective than OKT3.

Numerous experimental studies and a few clinical trials have explored the use of monoclonal anti–T cell antibodies that are more selective for subpopulations of T cells (448–451). Subset-specific antibodies such as those recognizing CD4 or CD8 have helped define the pathways of alloreactivity in animal models, but it is unclear whether knowledge of these pathways could be used to predict accurately which antibodies will be effective clinically and under what circumstances. Monoclonal antibodies to the α chain of the IL-2 receptor (CD25) have been evaluated in an effort to achieve greater antigen specificity with anti–T cell antibodies (452–456). Because CD25 is only transiently expressed when T cells are activated, such therapy might selectively eliminate only those T cells activated at the time of allogeneic challenge. Although clinical results with rodent anti-CD25 antibodies were disappointing, more recent studies suggest that humanized versions of anti-CD25 antibodies are more efficacious (457).

Monoclonal antibodies also have been used to block the effector mechanism of graft rejection. Anti-ICAM antibodies are immunosuppressive in monkeys and may function at the effector stage (434), and anti-TNF antibodies also have been tested experimentally. Agents that suppress T-cell costimulation via CD28 or CD40 ligand and anti-CD2 mAbs have shown promise, particularly when given in combination in animal models (458–460), and they are likely to be clinically evaluated in the near future.

Although mAb therapy has been extremely effective (461,462), several problems still exist. First, the interaction of the mAb OKT3 with the CD3 antigen initially activates T cells, stimulating release of several lymphokines, before the target cells are depleted (463). Some of these lymphokines, including TNF-α, can cause significant clinical side effects, including fever, chills, and pulmonary edema. These side effects usually can be well controlled and are seldom life threatening. Second, OKT3 and many of the mAbs that have been evaluated in humans are mouse proteins. When these are injected, human recipients generally respond in time with antibody production against both constant region and idiotypic determinants of the mAb (462). These antibody responses may hinder both repeated courses of therapy with the original antibody and treatment with other mAbs of different specificity, although higher dose therapy can usually overcome a recipient's antibody response. Third, mAb therapy may not always eliminate the target T-cell population. For example, during OKT3 treatment, cells of T-cell lineage expressing CD4 and CD8 antigens, but without the CD3 antigen, usually return to the circulation. This phenomenon is referred to as modulation of the target antigen (461). In the case of OKT3, the absence of the CD3 surface structure renders T cells immunologically incompetent (464). The loss of other surface antigens, however, may not be as immunosuppressive. Finally, OKT3 provides broad, nonspecific immunosuppression that renders the recipient significantly immunoincompetent. Although OKT3 does not cause permanent nonresponsiveness to environmental antigens, unfortunately neither does it achieve permanent tolerance to the antigens of the graft.

Many of the difficulties associated with mAb therapy are associated with the constant region of the antibody. For example, T-cell

activation does not occur when F(ab)'2 antibodies are used, and antiantibody responses by the recipient are much weaker in the absence of the constant region (465). Considerable effort has therefore been devoted to engineering ideal therapeutic antibodies. One approach has been to construct chimeric mouse antibodies with human Fc portions, or to graft the hypervariable (CDR3) component of a murine antibody to an otherwise human molecule. This latter process is known as humanizing an mAb from another species. Another promising approach has been to couple toxic elements, such as ricin or diphtheria toxin, to the antibody (466,467).

Donor-Specific Tolerance Induction

The Need for Tolerance-Inducing Regimens

Specific immunosuppression in transplantation immunology involves suppression of the immune response to donor allo- or xenoantigens but not to other antigens. The ultimate form of specific unresponsiveness is tolerance, in which the donor-specific nonresponsiveness is maintained permanently without further treatment. The achievement of transplantation tolerance has been the goal of transplantation immunology for over 40 years, for three major reasons. First, although improvements in immunosuppressive therapy have dramatically increased the success of clinical organ transplantation, these drugs are associated with life-long increased risks of infection and malignancy. Chronic drug treatment would not be required in tolerant recipients. Second, despite improved immunosuppression, chronic rejection is still a major problem and leads to constantly downsloping long-term survival curves for organ allografts. Chronic rejection would not occur in tolerant recipients. Third, a critical shortage of allogeneic organs has increased interest in the use of other species as xenogeneic donors. However, immune barriers to xenografts may be even greater than those to allografts (468), and the induction of both B-cell and T-cell tolerance may be essential to the ultimate success of xenotransplantation.

Central and Peripheral Tolerance

Other chapters in this book describe the mechanisms by which tolerance to self antigens is achieved (see Chapter 20). For developing T cells, these processes take place in the thymus, which is the central organ for T-cell development. Hence, induction of tolerance among developing thymocytes is referred to as "central," as distinguished from the peripheral tolerance that may develop among already mature T cells when they encounter antigen in the peripheral tissues.

B cells are also susceptible to tolerance induction by several mechanisms during development in the marrow. However, unlike T cells, the term "central tolerance" is not generally used in this context, largely because the marrow, the major organ for B-lymphopoiesis in mammals, is not dedicated exclusively to this activity.

Mechanisms of Transplantation Tolerance

The known mechanisms for inducing T and B cell tolerance can be grouped into the categories deletion, anergy, and suppression. In addition, a graft may simply be ignored by recipient lymphocytes. Each of these mechanisms will be described briefly below, and then particular strategies designed to capture these mechanisms to alter transplantation tolerance will be discussed.

Clonal Deletion

Recent studies have demonstrated that a major mechanism by which self tolerance normally develops among both B and T cells is deletion of developing lymphocyte with specificity for self antigens.

Studies using Ig receptor transgenic mice suggest that B cells are susceptible to deletion at particular stages of development upon recognition of membrane-bound antigen (469). Antigen expression on either radioresistant host cells or on a small population of hematopoietic cells appears to be sufficient to delete immature B cells specific for that antigen (470). Cells of the B1 subset, which are thought to be the major source of the natural antibodies responsible for xenograft hyperacute rejection, can be deleted by an apoptotic process in mice when their surface Ig is cross-linked by cell-bound antigen (471).

Deletion of self-reactive T cells is believed to occur when the avidity of an interaction between an immature thymocyte and an APC in the thymus is sufficiently high to induce apoptotic cell death (472,473). This high avidity is often due, at least in part, to a relatively high-affinity interaction between a rearranged TCR and a self peptide–MHC complex presented in the thymus. Several marrow-derived cell types, including dendritic cells (474), B cells (475), and thymocytes (476,477), as well as nonhematopoietic cells of the thymic stroma (371,478,479), have the capacity to induce intrathymic tolerance by both deletional and nondeletional mechanisms. Tolerance induced by intrathymic deletion should be reliable because the absence of lymphocytes with reactivity to the donor would ensure that a specific response to donor antigens could not be induced under any circumstances.

Peripheral deletion also has been described for mature T cells upon exposure to antigen *in vivo* (480–482). In addition, veto cells can delete alloreactive CTL precursors (CTLp).

Anergy

Anergy is another consequence that may result when T cells recognize peptide–MHC complexes, but without receiving adequate accessory or costimulatory signals. A lymphocyte is considered to be anergic if it cannot respond to APCs expressing the antigen for which it is specific. Frequently, in the case of T cells, the anergic state is associated with a lack of IL-2 production and can be overcome by providing exogenous IL-2 (373). Numerous methods of inducing T- and B-cell anergy have been described (469,483–486). In some cases anergy has been associated with TCR or Ig receptor downregulation (469,482,487–489). In the case of B-cell tolerance, the induction of anergy versus activation may be dependent on antigen concentration (469). T cells typically become anergic if they encounter antigen without costimulation, but they also may undergo anergy if they encounter peptide ligands for which they have low affinity (472,473). It appears that thymocytes (in addition to mature peripheral T cells) are also susceptible to anergy induction, particularly by antigens presented on thymic epithelium or dendritic cells (475). Anergy is generally reversible *in vivo* and can be overcome by infection (490) or by removal of antigen (491,492).

Suppression

A potential form of antibody-mediated suppression might be through the recognition of idiotypes of antidonor receptors. Idio-

types are unique antigenic determinants that characterize the binding sites of antibody or T-cell receptors and can therefore be detected by antiidiotypic antibodies (493,494). In the case of transplantation responses, such antiidiotypic reagents might have the potential to specifically modify responses to subsequent transplants. For this reason, the antiidiotype approach to specific immunosuppression has received considerable attention in the past. We now know, however, that the determinants recognized by B cells and the genes encoding B-cell receptors are different from those involved in T-cell responses. Thus, it is unlikely that cellular immunity can be modified by antiidiotypic reagents raised against B cells. It continues to be an intriguing question whether normal regulatory mechanisms for B-cell responses might include antiidiotypes, as suggested by Jerne. However, efforts to control transplantation using exogenous antiidiotypic antibodies to either T- or B-cell receptors have been disappointing (495).

Antibodies also can induce tolerance through a process known as enhancement. Enhancement is defined as prolongation of graft survival achieved by the presence of antigraft antibodies (496). This phenomenon was first described in experiments involving allogeneic tumor growth (497). Subsequently, Stuart et al. (498) and Batchelor (499) demonstrated that enhancing regimens using anti-MHC antibodies or soluble antigen could produce long-term tolerance for rodent allogeneic kidney transplants (500). The simple interpretation was that anti-MHC antibodies bind to the antigen and thereby block the immune response, but this explanation has not turned out to be sufficient. For example, tolerance after enhancement can be transferred by cells and not serum from enhanced recipients. Apparently, the administered antibody sets up a host reaction that leads to specific immunosuppression. An idiotype antiidiotype network would be an attractive explanation for this phenomenon. Unfortunately, the spectacular success obtained using enhancement for kidney graft survival in rats has not been observed for grafts in other species.

Another form of suppression promoting graft acceptance may be mediated by cell populations. Suppression of T-cell responses has been attributed to both T cells and non–T cells, and may show varying degrees of specificity. In some instances, nonspecific suppressive effector molecules may be secreted in response to a specific antigenic stimulus, thus conferring apparent specificity. In the ensuing discussion, we categorize mechanisms of suppression in terms of the degree of specificity of their ability to suppress transplant rejection.

Nonspecific Suppression. Nonspecific suppression can result from secretion by cells, in a non–antigen-specific manner, of soluble molecules that downmodulate immune function, such as cytokines (501,502), nitric oxide (503,504), and prostaglandins (505,506). Nonspecific suppressive cell populations such as natural suppressor (NS) cells may mediate suppression, at least in part, via soluble mediators (507–511). Obviously, nonspecific suppressive mechanisms would only be of interest for tolerance induction if they could be induced temporarily during a critical period when alloreactivity was present. In the absence of mechanisms for ensuring specific tolerance, nonspecific suppressive cells offer no advantage over other nonspecific immunosuppressive therapies. Facilitation of specific suppressor cell development by nonspecific, phenotypically null natural suppressor cells has been described (512–514).

Veto Activity. Veto activity, which is the ability to inactivate CTLp reacting against alloantigens expressed on the surface of the veto cell (515,516), confers suppression of CTL responses directed against any antigens shared by the veto cell. CTLs themselves (517), as well as activated NK cells (518) and poorly characterized cell types in hematopoietic tissues (515), have been found to have veto activity. Although the mechanism of vetoing is poorly understood, in some instances it may involve signaling through the MHC class I molecule of the CTLp upon ligation by CD8 expressed on the veto cells (519–521). However, not all veto cells express CD8, so this mechanism cannot explain all veto phenomena. Veto activity has been suggested to involve the immunosuppressive cytokine TGF-β (501).

Antigen-Specific Suppression. Although functional evidence for the existence of alloantigen-specific suppressor cells has been obtained in several transplantation models (450,522–529), such cells have been cloned in only a few instances (530), and suppressor cell–specific surface markers have not been identified. In addition, mechanisms of specific suppression of alloreactivity have not been well characterized. In several instances, antiidiotypic T-cell recognition by other T cells has been implicated in mediating specific suppression of graft rejection or graft-versus-host responses (523,526,529).

It is possible that some antigen-specific suppressive phenomena might be explained by the recently described dichotomy of T-helper cell function (531). A shift to the IL-4– and IL-10–producing T-helper type 2 (Th2) type of response from a proinflammatory Th1 (IL-2– and IFN-γ–producing) response has been associated with allograft acceptance (532–537). Because Th1 and Th2 responses mutually downregulate one another, these observations have led to the hypothesis that Th2 responses are tolerogenic. Th2 responses may influence APCs in a manner that prevents them from providing costimulation that could cause naive T cells to differentiate into Th1 cells. Thus, the APC might serve as an important intermediary for this and other infectious forms of tolerance. For practical purposes, an immune response that is nondestructive and inhibits the development of destructive responses could provide a powerful means of ensuring graft acceptance. However, most of the data merely demonstrate an association of Th2-dominant responses with graft acceptance, and only limited data exist to implicate an active role for Th2 cells in tolerance induction (538,539). It is clear that Th2 responses are not always benign because Th2 cells and Th2-associated cytokines can mediate or contribute to allograft rejection (540–543). It has recently been suggested that the xenograft reaction in at least one species combination is associated with a predominant Th2-type response (544). However, Th2-associated cytokines also have been detected in accepted xenografts in which accommodation had occurred (146). Furthermore, the Th1/Th2 dichotomy of cytokine production is not always clear-cut, and future studies are likely to better clarify the role of individual cytokines in inducing graft acceptance and rejection. Recently, a subset of murine and human $CD4^+$ cells that produces IL-10 but not IL-4 has been reported to suppress *in vitro* responses as well as inflammatory bowel disease *in vivo* (545).

Lymphocytes Ignoring Graft Antigens (Ignorance)

Experimental situations have been described in which antigens are ignored by T cells (489,546,547) or B cells (469) that can recognize them. This may be due to the presentation of these antigens by nonprofessional APCs that are unable to activate T cells, or it may reflect a failure of recipient T cells to migrate to the graft tissue and encounter donor antigens. The level of peripheral antigen

expression, how recently the responding T cell has emerged from the thymus (489,548), and the presence of proinflammatory cytokines (549,550) and upregulated costimulatory molecules within peripheral tissues (551) all may influence the decision of a T cell to ignore or respond to peripheral antigens. However, in contrast to a state of systemic tolerance, ignorance may be a more precarious state that can be upset by additional immunologic stimuli provoked by inflammation that may be induced, for example, by infections (547) or by presentation of antigen on professional APCs, as has been described for endocrine allografts that are depleted of APCs before transplantation (552).

Strategies for Inducing Transplantation Tolerance

Strategies to Achieve Central Tolerance

Mixed Chimerism. The pioneering work of Owen, Medawar, and others, beginning 50 years ago, led to the observation that hematopoietic chimerism can be associated with a state of donor-specific tolerance (553). The capacity of hematopoietic cells to induce tolerance results largely from their ability to induce intrathymic clonal deletion of thymocytes specific for antigens expressed by the hematopoietic cells. Thus, bone marrow engraftment can reliably induce tolerance to the most immunogenic allografts, such as fully MHC-mismatched skin grafts and small bowel grafts (155,554). In view of this powerful tolerance-inducing capacity, it may seem surprising that hematopoietic cell transplantation has not yet been applied to the induction of tolerance in humans. However, bone marrow transplantation (BMT) for tolerance induction has traditionally involved recipient treatment with lethal whole-body irradiation to eliminate mature recipient T cells and to make hematopoietic space for the marrow allograft. Removal of mature donor T cells before transplantation can prevent graft-versus-host disease (GVHD) (555–558). Under these circumstances, a new immune system can develop that is tolerant to both donor and recipient antigens. Although this approach has been successful in rodents, MHC-mismatched allogeneic BMT in larger animals, including humans, has proved to be less successful and more dangerous because of the unacceptable toxicity associated with myeloablative conditioning, and the inordinately high risks of GVHD and engraftment failure (559). Therefore, it will be useful to develop more specific and effective methods of overcoming the barriers to marrow engraftment. Achievement of this goal will require an understanding of the immunologic and physiologic obstacles to the engraftment and function of allogeneic and xenogeneic hematopoietic cells. It would be most desirable to achieve a state of mixed hematopoietic chimerism rather than full donor reconstitution in completely MHC-mismatched combinations. Improved immunocompetence has been observed in mixed chimeras, which contain host-type APCs in the peripheral tissues, presumably because these allow optimal antigen presentation to T cells that have developed in the host thymus, and which may therefore preferentially recognize peptide antigens presented by host-type MHC molecules (560,561).

Several approaches have been developed to permit the use of BMT to achieve mixed chimerism and specific tolerance. The use of total lymphoid irradiation (TLI) plus BMT has been studied extensively in rodents (562,563). The long bones are shielded during the radiation preparative regimen, so BMT results in mixed chimerism rather than full donor reconstitution. Mice treated in this way have been shown to be both resistant to GVHD and tolerant to skin grafts from donor but not third-party animals. The mechanism by which TLI induces this tolerance is incompletely understood and its success varies depending on the species involved. In rodent models, Th2-type responses appear to predominate when antigens are introduced after TLI, and this Th1 to Th2 shift may play a role in permitting graft acceptance (564,565). Natural suppressor cells that inhibit IL-2 production also have been implicated in tolerance induction after TLI (566). In clinical transplantation, TLI also has been used successfully, although it is cumbersome, especially in the case of cadaver donor transplantation (567,568). Donor-specific tolerance has been demonstrated in a small number of patients in whom immunosuppressive therapy was terminated after kidney transplantation under cover of TLI (569). Posttransplant TLI has been shown to have beneficial effects in conjunction with antithymocyte globulin in a primate model (570).

Mixed chimerism also can be achieved in rodents by administering a combination of T cell–depleted syngeneic and allogeneic bone marrow cells to lethally irradiated recipients. Mixed bone marrow chimeras are fully immunocompetent (560) and are specifically tolerant to subsequent skin grafts from the donor (571,572). More recently, mixed chimerism and donor-specific tolerance across MHC barriers has been achieved in mice without myeloablative conditioning (155). This model involves host treatment with depleting anti–T cell mAbs followed by a sublethal dose of whole-body irradiation to create hematopoietic space and a higher dose of local irradiation to the thymus to eliminate thymocytes that are not depleted by the mAbs. Several other regimens involving various combinations of anti–T cell antibodies, irradiation, and immunosuppressive drugs also have permitted the achievement of mixed chimerism in both large (573) and small (574–577) animals. The successful induction of tolerance in a primate model using the nonmyeloablative approach to inducing mixed chimerism (573) has been used as the basis for a pilot clinical trial.

Mouse studies involving the use of mAbs to permit marrow engraftment have helped to elucidate the immune barriers to such grafts (155,578,579). As expected, $CD4^+$ cells resist engraftment of class II–mismatched marrow, and CD8 cells reject class I–disparate marrow grafts. However, $CD8^+$ cells also mount significant resistance to class II–mismatched marrow, and $CD4^+$ host cells pose a weak but detectable barrier to class I–mismatched marrow (578,579). Although NK cells resist allogeneic myeloid progenitor cell engraftment, as is described above, they actually present only a weak barrier to the engraftment of allogeneic long-term multilineage repopulating, pluripotent hematopoietic stem cells (PHSCs) (153,580). However, NK cells may play a more significant role in resisting the engraftment of xenogeneic marrow (154).

Gene Therapy of Autologous Marrow for the Induction of Tolerance. An alternative to using allogeneic hematopoietic engraftment to achieve tolerance is to reconstitute recipients with autologous bone marrow cells that have been transfected with genes encoding foreign transplantation antigens. This approach has permitted markedly prolonged survival of class I–disparate skin grafts bearing the class I gene that was introduced into the autologous marrow (581). If the genes selected encode particularly important antigens (such as those determining class II antigens), then tolerance to these antigens may have a significant effect on the rejection of subsequent grafts expressing these and other transplantation antigens and can lead to transplantation tolerance (582).

Extension of the Mixed Chimerism Approach to Xenotransplantation. Host treatment with mAbs to T cells and NK cells along with sublethal irradiation also has permitted rat marrow engraft-

ment in mice, resulting in mixed xenogeneic chimerism and donor-specific tolerance (154). However, hematopoietic function depends on interactions between adhesion molecules and their ligands (583) and on a number of specific molecular interactions between the stroma and hematopoietic cells. It is probably the species specificity of some of these interactions (cytokines, adhesion molecules, other cell surface signaling molecules) that accounts for the competitive advantage enjoyed by recipient marrow over xenogeneic rat marrow that becomes increasingly evident as recovery of the host from low-dose whole-body irradiation occurs (584,585).

Achievement of xenogeneic hematopoietic repopulation has proved to be an even more formidable challenge in more disparate species combinations. Human and pig progenitor cells have been shown to be capable of repopulating murine recipients at low levels (586–588), but the species specificity of critical regulatory molecules may limit the level of donor repopulation. Administration of exogenous donor species-specific cytokines can partially overcome this barrier (587,589).

Xenogeneic Thymic Transplantation. Because of the difficulties encountered in inducing xenogeneic hematopoietic cells to migrate to a recipient thymus and induce central tolerance, an alternative approach might involve replacement of the recipient thymus with a xenogeneic donor thymus after host T-cell depletion and thymectomy. Immunocompetent mice treated in this way demonstrate recovery of $CD4^+$ T cells in xenogeneic porcine thymic grafts (590). These cells repopulate the periphery, are competent to resist infection (591), and are tolerant of donor antigens, even by the stringent measure of discordant xenogeneic skin grafting (592). Tolerance to both donor and host develops, at least in part, by intrathymic deletional mechanisms in these animals, and this reflects the presence of class II^{high} cells from both species within the thymic graft (592,593). Because MHC restriction is determined by the MHC of the thymus, it is surprising that T cells that differentiated in a xenogeneic thymus are able to respond to peptide antigens presented by host MHC (591). However, the excellent immune function achieved in humans receiving human leukocyte antigen (HLA)-mismatched allogeneic thymic transplantation for the treatment of congenital thymic aplasia (DiGeorge syndrome) suggests that this restriction incompatibility may not be a major obstacle to the achievement of adequate immune function (594). Perhaps this high level of cross-reactivity, even between species, reflects the fact that MHC reactivity is inherent in germline TCR sequences (51).

Transplantation of allogeneic (595) and concordant xenogeneic (596) thymic tissue obtained from fetuses before the time that hematopoietic cells have seeded the organ can also induce a form of tolerance that permits skin graft acceptance. However, the mechanism of tolerance in such animals is unlikely to be deletional because donor-specific MLRs are preserved. Active suppression has been implicated in the allogeneic model (597,598), and studies in the pig to mouse thymic transplantation model described above also suggest a possible role for suppression.

Development of Chimerism and Tolerance Without T Cell–Depleting or Myelosuppressive Treatment. Developmentally immunoincompetent recipients. In theory, fetuses might be permissive for engraftment of allogeneic hematopoietic stem cells, not only because of their immunologic immaturity, but because space might be available in their hematopoietic systems, so that engraftment could be achieved without host conditioning. Since prenatal diagnosis of a number of congenital diseases has become possible, there is renewed interest in the possibility of injecting allogeneic or xenogeneic pleuripotential hematopoietic stem cells (PHSC) into preimmune fetuses. Intrauterine injection of allogeneic hematopoietic stem cells has been used successfully to correct immunodeficiency diseases diagnosed in utero in human fetuses (599,600). However, chimerism was only detected in the T cell compartment afflicted by the congenital deficiency, and not in other hematopoietic lineages. In view of this result and the observation of only low levels of chimerism in preimmune normal mouse fetuses and sheep receiving in utero transplants (601), the concept that hematopoietic space is present in preimmune fetuses is open to question. In a large animal model, successful engraftment of enriched human PHSC populations has been successfully achieved without GVHD (602), raising hopes that this approach could be used for a broader spectrum of disorders in human fetuses. However, the ability of in utero marrow transplantation to induce tolerance is somewhat unpredictable (601,603).

Untreated neonatal rodents can be rendered tolerant of alloantigens by the administration of allogeneic hematopoietic cells shortly after birth. The mechanisms responsible for this phenomenon of neonatal tolerance are slowly being unraveled. Lasting microchimerism has been detected in some neonatally tolerized mice, and evidence to support an intrathymic deletional mechanism has been obtained in some, but not other, strain combinations (604). Furthermore, the presence of microchimerism does not always predict skin graft tolerance in recipients of allogeneic lymphocytes perinatally, and nontolerant animals can still maintain microchimerism after rejection of donor skin grafts (605,606). Thus, it is not surprising that several additional mechanisms have been implicated in rodents in which neonatal tolerance has been induced. First, tolerance cannot be easily broken by the infusion of nontolerant host-type lymphocytes in neonatally tolerized animals (523,604,607), and this has been attributed to the presence of suppressive T-cell populations (523). The ability of neonates to mount host antigraft responses may be essential for the induction of these suppressive cell populations when donor antigen is given. In contrast, when deletional tolerance is induced in animals in which the preexisting peripheral T-cell response has been fully ablated (e.g., mixed chimeras prepared with the lethal or nonlethal regimen described above), the absence of suppressive cell populations makes it easy to abolish tolerance by the infusion of nontolerant host-type lymphocytes (608,609). Second, neonatal mice have a tendency to produce Th2 responses, and these have been implicated in donor-specific skin graft acceptance (537,610). However, neonatal mice are capable of mounting CTL and Th1 responses under certain conditions (611,612). Third, the ability of allogeneic spleen cell infusions to induce tolerance has been suggested to reflect the high ratio of non-costimulatory APCs (T and B cells) in donor inocula to recipient T cells in the neonate, rather than to any unique susceptibility to tolerance induction (613).

Adult recipients. Based on the recent observation that microchimerism can exist for many years in the tissues of human recipients of solid organ allografts who did not receive hematopoietic cell transplants (614), it has been hypothesized that microchimerism, resulting from emigration of passenger leukocytes from the graft to recipient tissues, leads to a state of donor-specific tolerance (615). However, this hypothesis is controversial (616), and it is currently unclear whether chimerism induces tolerance or is even an epiphenomenon that reflects either tolerance or adequate immunosuppressive pharmacotherapy. There are several mechanisms by which microchimerism, might in theory induce peripheral T-cell tolerance. These include nonprofessional APC function of donor-derived B or T cells (617–619), which can toler-

ize responding T cells. In addition, veto activity of T cells, NK cells, and other cell types eliminates CTLs reactive against antigens expressed on the veto cells (620). In addition to these mechanisms by which chimerism might induce peripheral tolerance, donor leukocytes migrating to recipient thymus might induce central tolerance among T cells that develop subsequent to the time of donor engraftment. Recent evidence shows that adult rodent liver grafts contain self-renewing hematopoietic stem cells (621,622), and dendritic cell progenitors have been detected in the marrow of mice that spontaneously accept mouse liver allografts (623). However, lasting microchimerism does not appear to play a role in several animal models of tolerance in which the issue has been carefully examined (624–626). It also has become increasingly clear in humans that microchimerism neither denotes a state of tolerance nor is required to maintain an allograft under all circumstances (616,627–629).

Recently, several groups have evaluated the ability of donor bone marrow cell infusions given without recipient myelosuppression or T-cell depletion to enhance graft survival. Although it is too early to determine whether this approach will be beneficial (630,631), it is clear that such transplants can be associated with significant risks from possible immunosuppressive effects of the transplant and from GVHD (632–634). These studies did not include intentional peripheral T-cell depletion of the hosts. In a primate model that includes recipient pretreatment with antilymphocyte serum for T-cell depletion, and, for optimal results, total lymphoid irradiation, veto cells in donor bone marrow that inactivate recipient CTLp may promote graft acceptance (501). However, only a fraction of recipients show long-term graft acceptance, with the best results obtained when the donor and recipient share a DR class II MHC allele (635).

In the primate BMT model described in the preceding paragraph, no myelosuppressive treatment was included in the host conditioning regimen, and, not surprisingly, only low levels of chimerism were detected. In contrast, macroscopically detectable chimerism has been observed in an otherwise similar primate model that includes a sublethal dose of host irradiation (573). The concept that myelosuppression must be used to create space in the hematopoietic compartment in order to allow donor hematopoietic cells to engraft has long been widely accepted. The mechanism by which myelosuppression promotes marrow engraftment is not fully understood, and could include both the creation of physical niches due to the destruction of host hematopoietic cells, and the upregulation of cytokines that promote hematopoiesis. In syngeneic BMT recipients, a low dose of whole-body irradiation is required to make physiologic space for engraftment of syngeneic marrow cells given in numbers similar to those that could be obtained from marrow of living human allogeneic marrow donors (636). However, this requirement can be overcome by the administration of high doses of syngeneic marrow (637,638). Furthermore, engraftment of high doses of allogeneic marrow can be achieved without myelosuppressive treatment in mice that receive T cell–depleting mAbs (639). However, it is essential to create space in the thymus and to achieve high levels of early donor T-cell repopulation in order to induce permanent skin graft tolerance (639). Apparently, thymic space and peripheral hematopoietic space are regulated independently (638).

Strategies to Achieve Peripheral Tolerance

There are numerous animal models in which peripheral T-cell tolerance has been induced, generally by mechanisms that are not fully understood. Evidence for anergy as a mechanism of peripheral tolerance has been achieved in several of these models, including transplantation of I-E islet allografts into I-E$^-$ recipients after anti-CD4 mAb treatment (451), and in mice receiving allogeneic BMT after treatment with T cell–depleting mAbs (640).

Costimulatory Blockade. Other attempts to induce anergy in transplantation models have included the introduction of alloantigen on non-costimulatory APCs, such as cells whose antigen-presenting activity has been impaired by ultraviolet irradiation (641). It is possible that donor-specific transfusions facilitate tolerance induction (642) because of their B-cell and-T cell contents, both of which can present antigen in a manner that induces anergy (618,643). Efforts to induce tolerance by blocking the B7/CD28 costimulatory pathway have enjoyed success in animals receiving vascularized allografts (644–647) or pancreatic islet xenografts (648) under cover of CTLA4Ig, a synthetic soluble molecule that contains the B7-binding portion of CTLA4, one of the T-cell ligands of B7. CTLA4Ig blocks CD28 binding to both B7 molecules, B7-1 and B7-2. This appears to combine with the tolerogenic capacity of primarily vascularized organ allografts in rodents (113,649–652). The ability of differential blocking of B7-1 or B7-2 with specific mAbs to selectively drive Th1-type or Th2-type T-cell responses is controversial (653,654), and the distinct functions of each of these B7 molecules have not been clearly defined. Evidence in a rat renal allograft model suggests that CTLA4Ig may favor the development of Th2 responses, rather than inducing anergy (646).

Recent studies have demonstrated the importance of interactions between CD40 ligand (CD40L) on T cells and CD40, which is expressed not only on activated B cells, but on a variety of APCs. Signaling through the interaction of CD40 on APCs with its T-cell ligand is necessary for the conversion of certain nonprofessional APCs, such as B cells, to functional APCs, in part by upregulating B7 expression. In addition to its important role in providing cognate help for B-cell activation and in activating APCs, this interaction provides important costimulation leading to T cell activation. Blocking CD40L/CD40 interactions can result in graft prolongation that may be associated with a Th2-dominant immune response or with a reduction in the production of nitric oxide, an important mediator produced by activated macrophages (376,655,656). The combination of CTLA4Ig and anti-CD40L antibody seems to provide particularly potent immunosuppression with the ability to markedly prolong the survival of primary skin allografts (459,460). The role of anergy, if any, in these systems, remains to be determined.

T-cell anergy also has been induced in transplantation models by the administration of mAbs that block adhesion molecules (553). Combined treatment with mAbs against LFA-1 and its ligand ICAM-1 has been reported to induce profound tolerance in murine cardiac allograft recipients (657).

Nondepleting Anti–T Cell Antibodies. Th2 cytokines may play a role in the induction of infectious tolerance observed in mice receiving minor antigen-disparate skin allografts under cover of nondepleting anti–T cell mAbs (658–660). Waldmann and colleagues have demonstrated that CD4$^+$ T cells, rendered incapable of rejecting the allograft in the original recipients, could render naive T cells unresponsive in secondary recipients, and that these tolerant T cells could, in turn, tolerize naive T cells in tertiary recipients. However, a Th1 to Th2 shift may not fully account for the tolerance in the above model (659).

A Large Animal Model of Peripheral Tolerance Induction

Studies of pig renal transplantation have demonstrated that spontaneous tolerance can be induced by organ grafts in large animals, provided that MHC antigens are matched (40). The ability to achieve such tolerance is dependent on one or possibly two non–MHC-linked genetic loci in the recipient animals (494). The presence of the acceptor phenotype also permits the spontaneous acceptance of single haplotype class I–mismatched kidney grafts (40). Graft acceptance is associated with donor-specific CTL unresponsiveness, apparently due to a deficiency in help for these CTLs, and not due to a deletional mechanism (105). The requirement that class II antigens be matched between donor and recipient in order for this tolerance to be achieved may reflect the influence of a major difference in class II antigen expression that exists between large and small animals. Unlike large animals and humans, in which class II antigens are expressed constitutively on vascular endothelial cells (44), the corresponding endothelial cells of rodent species do not express MHC class II molecules, even in the setting of allograft rejection (37,44). Consistent with this interpretation, the use of a short course of cyclosporine A (CyA) can facilitate the ability of renal allografts to induce tolerance in rodents (661,662) across fully MHC-mismatched barriers, but tolerance induction in swine requires class II matching between donor and recipient for uniform success (663). Thus, in class II–matched, class I–mismatched porcine donor–recipient pairs, a 12-day course of high-dose (10 mg/kg/day) CyA permitted long-term graft acceptance in 100% of cases (663), and animals accepting such allografts became systemically tolerant to the donor's class I and minor antigens, as indicated by the fact that the accepted graft could be removed and replaced by a second donor-matched graft, which was likewise accepted (664). This ability of CyA to facilitate tolerance induction, and the ability of exogenous IL-2 to prevent the induction of tolerance in this model (664), are consistent with the interpretation that induction of tolerance of donor class I–reactive CTLs is due, at least in part, to the absence of adequate T-cell help during the time of initial exposure to antigen. Also consistent was the selective decrease of expression of the Th1-associated cytokine IFN-γ relative to the Th2-associated cytokine IL-10, which has been observed in these accepted grafts (665,666). The thymus appears to play a role in the induction of tolerance among preexisting peripheral T cells in this model because removal of the host thymus before kidney allotransplantation leads to rejection (667). The possible mechanisms responsible for this role of the thymus in inducing peripheral tolerance phenomenon are discussed elsewhere in this chapter.

The Relationship Between Peripheral T-Cell Tolerance and Central Tolerance

The distinction between central and peripheral tolerance may not always be clear. In some systems, passenger leukocytes might emigrate from the graft to the host thymus and tolerize subsequently developing thymocytes. In addition, however, the thymus may be capable of tolerizing T cells that were already in the periphery at the time of organ grafting. In the pig model described above, the thymus appears to play a role in the induction of tolerance among preexisting peripheral T cells (667). It is possible that T cells that are activated in the periphery by the organ allograft recirculate to the thymus as has been described (668) and encounter donor antigen there in ways that inactivate the T cells. This could be a mechanism for ensuring that T cells activated in the periphery of an animal are switched off if the same antigens are present on intrathymic leukocytes. Alternatively, the migration of donor antigen to the thymus may result in the development of T cells that specifically recognize the donor antigen and downregulate the activity of destructive alloreactive T cells when they enter the periphery (522,665).

A second situation in which the boundary between central and peripheral tolerance is blurred arises when donor antigens are injected intrathymically in order to induce tolerance. The initial idea underlying this approach was to use antibody treatment to deplete peripheral T cells and to induce central tolerance among recovering T cells by direct introduction of antigen into the thymus. However, more recent studies have shown that tolerance to soluble alloantigens can be induced by intrathymic injection without peripheral T-cell depletion (669). Because removal of the thymus before or within the first few days of allografting results in rejection of the allograft (255), the thymus must play an active role in tolerizing preexisting peripheral T cells, possibly by one of the mechanisms proposed in the preceding paragraph. Active regulatory cell populations have been reported in rats receiving intrathymic injections of allogeneic bone marrow cells (BMC) (670). These results are consistent with the role of suppressive cell populations in tolerance induced by thymic allografts (597,598) or xenografts.

There is an important role for the allograft in inducing tolerance in animals receiving intrathymic injection, and in other models in which a preexisting peripheral T-cell repertoire must be rendered tolerant. Transferable tolerance is not induced by intrathymic marrow injection alone without an organ allograft in rats (670), suggesting that the graft itself helps to tolerize the preexisting T-cell repertoire. In contrast, pure intrathymic deletional tolerance is not dependent on the continued presence of antigen in the periphery (609). Donor tissue can be grafted at any time, and tolerance is assured. The intrathymic injection approach has not been successful in high responder rat strain combinations and may even induce allosensitization (671,672), and has not successfully allowed xenotolerance induction.

Which Strategy to Achieve Transplantation Tolerance?

Although clinical trials have already begun in which tolerance-inducing strategies are combined with conventional pharmacologic immunosuppression, none of the strategies for achieving transplantation tolerance have been used to replace such chronic therapy in clinical transplantation. In general, short-term results of most organ allograft transplants are excellent, making it essential to have extremely reliable methods of inducing tolerance in order to ethically justify their use in place of conventional chronic immunosuppressive therapies. Although induction of central deletional tolerance with hematopoietic cell grafts is a reliable and durable approach to achieving permanent graft survival, earlier techniques for achieving central tolerance have involved more vigorous ablation of the lymphohematopoietic system than can be safely achieved in larger animals. Thus, the major challenge in bringing this approach to clinical application is to develop highly specific, nontoxic methods of conditioning the host for acceptance of a hematopoietic allograft or xenograft. On the other hand, techniques to achieve peripheral tolerance in larger animals have not generally produced as good results as have been seen in rodent models. Fur-

thermore, peripheral mechanisms alone have not been sufficient to reliably overcome the most stringent transplantation barrier imposed by fully MHC-mismatched primary skin allografts. Conceptually, one major problem with peripheral tolerance strategies is that they cannot prevent the generation of new T cells in the recipient capable of recognizing donor antigens. Although effector cells might be persistently driven toward anergy or deletion by the nonstimulating cells of the graft, the ongoing stimulation of helper cells by professional APCs through the indirect pathway would seem likely to render this a precarious state of tolerance. The only means of completely eliminating the indirect pathway may be through central tolerance induction to the peptides of donor antigens presented in the thymus in association with host MHC antigens. However, thymic APCs appear to be incapable of picking up and re-presenting antigens from lymphoid cells to induce tolerance of T cells that recognize these antigens through the indirect pathway (477). Because superantigens have been shown to be capable of inducing deletional tolerance in the thymus when presented by APCs other than those that produced them (673), further definition of the circumstances under which indirect presentation of hematopoietic cell-derived antigens leads to intrathymic deletion is needed. In addition, the infectious nature of suppressive mechanisms of tolerance makes them potentially attractive as a means of inducing robust and durable tolerance. However, it will be difficult to control the development of such mechanisms until they are better understood. It seems likely that the optimal approach to achieving clinical transplantation tolerance might require combinations of both central and peripheral strategies.

TRANSPLANTATION OF SPECIFIC ORGANS AND TISSUES

Skin Grafting

Although allogeneic skin grafting represents a frequently used experimental model (674), its application in humans is unusual. Most skin transplantation in humans is performed with autologous tissue. Recently, however, artificial skin grafts have been created that consist of stromal elements and cultured cells of allogeneic and even xenogeneic origin. Evidence suggests that these grafts are not rejected, although their components may be replaced by recipient tissues over time.

Skin grafts are frequently used experimentally on small animals to examine rejection because they can be performed rapidly in large numbers. On the one hand, the use of skin grafts has the disadvantage that they are not primarily vascularized and, thus, may not be susceptible to precisely the same mechanisms of rejection as are solid organs. On the other hand, the difficulty in prolonging skin graft survival in rodents more accurately reflects the difficulty in prolonging transplantation of solid organ survival in larger animals than does the transplantation of solid organs in rodents.

Kidney Transplantation

Kidneys have been the most frequently transplanted organs for many years. At present approximately 10,000 kidney transplants are performed annually in the United States. The likelihood that a renal allograft will survive with good function for at least 1 year has slowly been increasing. Patient survival after 1 year is expected to be better than 90%, and the current likelihood of graft function at 1 year now exceeds 85% in many units, even when organs from totally unrelated donors are used.

Nonetheless, even well-matched recipients of renal transplants must continue to take immunosuppressive medications for the rest of their lives. These patients are susceptible to the complications of their immunosuppressive medications, including increased risks of infection, cancer, hypertension, and metabolic bone disease. Thus, they pay a price for their new organ stemming from our inability to provide specific immunosuppression. The success of clinical transplantation is a double-edged sword. With such good patient and graft survival rates initially, it is difficult to justify risky clinical trials of new approaches to immunosuppression that by achieving antigen-specific tolerance might avoid the long-term need for immunosuppression altogether.

Because of the success of modern nonspecific immunosuppression, the major obstacle to achieving a successful kidney transplant is no longer the rejection of the organ after transplantation. Instead the two major obstacles are now the shortage of organs and the problem of sensitization. Partly because of the increasing success of renal transplantation, the number of candidates for the procedure has continued to grow and now exceeds the supply of available organs. Unlike hearts and livers, where an inadequate supply of organs leads to the death of many candidates, those waiting for renal transplants are instead faced with long periods on dialysis. This waiting time is often 3 or more years even for unsensitized candidates seeking kidneys from cadaver donors. The second major obstacle in obtaining a successful renal transplant stems from the problem of sensitized candidates with broadly reactive antibodies resulting from prior antigen exposure. These highly sensitized individuals may wait many years to obtain a kidney that is cross-match negative, and some never receive a transplant at all.

Liver Transplantation

Transplantation of the liver represents a major technical challenge. For this reason the organ and patient survival rates are not as good as those for renal transplantation. However, successful liver transplantation can now be achieved with survival of about two thirds of the recipients at 1 year (675,676).

From an immunologist's point of view, liver transplantation is of interest first because the organ is apparently quite resistant to immediate antibody-mediated rejection (677). Transplantation across blood group barriers and in the face of a positive crossmatch (by retrospective analysis) has generally been successful in the short term (186). There is evidence, however, that long-term organ survival is diminished in blood group–incompatible patients (187). Second, the long-term survival of liver transplants does not appear to be better when better HLA matching between donor and recipient is achieved. In fact, some data have suggested the opposite correlation. The possibility that poorly matched livers may survive better than well-matched ones might be due to an inability of recipient T cells, sensitized to viral pathogens in association with self MHC antigens, to recognize those pathogens in the donor liver presented in association with donor HLA antigens. Thus, poorly matched livers might escape injury caused by immunologic responses to hepatotrophic viruses. Third, transplantation of the liver carries with it large numbers of donor lymphoid cells, thus creating the setting for GVHD. Donor lymphocytes can mediate an antibody-dependent hemolysis of recipient red blood cells in the case of recipient blood group incompatibility with the donor (678).

Thus A or B recipients of O livers have been subject to an immune hemolytic anemia during the early posttransplant period. There also may be other manifestations of GVHD even in blood group–compatible recipients (679).

Heart and Lung Transplantation

Heart transplantation is also a relatively recent component of standard clinical transplantation, with survival rates frequently in excess of 80% at 1 year (680,681). One of the immunologic issues of particular importance in heart transplantation is the high rate of new atherosclerotic disease in the coronary arteries of the donor organ (682). This atherosclerotic disease is probably a manifestation of chronic rejection.

Lung transplantation, either in conjunction with heart transplantation or alone, is a still more recent addition to clinical transplantation (683). Recipients of lung transplants have demonstrated a tendency to develop pathologic changes of bronchiolitis obliterans, which is thought to be a manifestation of chronic rejection.

Pancreas and Islet Transplantation

Transplantation of the whole pancreas met with almost 100% failure until about 1980, largely for technical reasons. More recently, successful pancreas transplantation to treat diabetes mellitus has been achieved using new technical approaches, and with success rates approaching those for kidney transplantation, as long as the two organs are transplanted together (684). The lower survival rates achieved when pancreas transplantation is performed alone probably reflects the difficulty in diagnosing rejection episodes involving this organ. By the time blood sugar levels begin to increase, destruction of the pancreas is generally so far advanced that it cannot be reversed by immunotherapy. Measurement of serum creatinine, reflecting early dysfunction of a simultaneous kidney transplant, allows much earlier detection of rejection activity and, thus, better outcomes (685). On the other hand, simultaneous transplantation of both a kidney and a pancreas from a single donor has demonstrated the interesting phenomenon that rejection activity in one organ is not always associated with rejection activity in the other (686). It is not known whether this occasional dichotomy reflects tissue-specific antigens or localized inflammatory events in one, but not the other organ.

Transplantation of the whole pancreas provides, of course, more tissue than is needed to treat diabetes mellitus. The intriguing aspect of pancreas transplantation, therefore, is the potential that useful results might be accomplished by transplantation of insulin-producing islet cells alone (422). Although islet cell transplants have been successful in animal models, success in humans has been limited (687). Part of the reason that islet transplantation has been so unsuccessful appears to be the autoimmune state of diabetic recipients in addition to their alloreactive response to the transplanted tissue. Thus, patients without previous diabetes, who have undergone islet transplantation at the time of total pancreatectomy, have had much higher success rates than have diabetic patients.

Even if islet transplantation could be performed routinely with current immunosuppressive drugs, it would not dramatically change the course of diabetes for these patients. This is because the primary goal of islet transplantation is to prevent the secondary neurologic, vascular, and retinal complications of diabetes that take many years to develop. However, performance of islet transplantation early in the course of the disease, when it might really affect these processes, would require exchanging insulin therapy for immunosuppressive drugs. Over 20 to 30 years, the latter is at least as damaging to human beings. Thus, even more than for other forms of transplantation, realization of the full potential of islet transplantation will require tolerance induction.

Hematopoietic Cell Transplantation

Bone marrow transplants, and more recently, transplants of hematopoietic stem cells and progenitors mobilized from the marrow into peripheral blood by treatment with cytokines, are used most commonly for the treatment of otherwise incurable leukemias and lymphomas, and for congenital immunodeficiency states. Although autologous stem cell transplants are currently used quite widely in the treatment of malignancies, these will not be considered further here because they do not involve the broaching of any immunologic barriers.

One fundamental difference between hematopoietic cell transplantation and the transplantation of all other organs is that the recipient's treatment for his or her malignancy usually results in ablation of the immune and hematopoietic systems before transplantation. Originally, stem cell allografts were administered only as a means of replacing these ablated host functions. Transplantation for immunodeficiency states does not require such ablation in order to achieve engraftment of allogeneic marrow grafts that reconstitute only the deficient immune system and not other hematopoietic lineages. Another major difference between BMT and solid organ transplantation is that the recovering immune system in BMT recipients is tolerant to the donor alloantigens, so there is no requirement for immunosuppressive therapy to prevent allograft rejection once the initial immune resistance to the allograft has been overcome. A third unique feature of BMT (as well as transplants of other organs that are rich in lymphoid tissue, such as small intestinal grafts and, to a lesser extent, liver grafts) is the ability of T cells in the allograft to mount an immunologic attack on the recipient's tissues, resulting in the condition known as GVHD. Although GVHD rates can be reduced to acceptable levels using prophylaxis with a course of nonspecific immunosuppressive therapy when the donor and recipient are HLA-matched siblings, the frequency and severity of the GVHD that develops when extensive HLA barriers are traversed has essentially precluded the routine performance of such transplants, making BMT unavailable to many for whom no other curative treatment exists. Recently, the establishment of large marrow donor registries has permitted the performance of matched transplants from unrelated donors in a significant fraction of patients, but these transplants are also associated with a high incidence of severe GVHD, due in large part to the existence of HLA mismatches that went undetected by conventional serologic HLA typing techniques.

Bone marrow transplantation for leukemia therapy was originally conceived as a way of providing hematopoietic rescue for patients receiving ablative cytoreductive treatments. However, one of the major benefits of the procedure has proven to be the graft-versus-host immune response, i.e., the recognition by donor T cells of host alloantigens, which are also expressed on leukemic cells. Thus, allogeneic BMT also may be thought of as immunotherapy leading to an attack on the residual leukemic cells that remain in cytoablated hosts. It is not surprising, therefore, that T-cell deple-

tion of the donor has not proved to be an optimal solution to the GVHD problem, primarily because the decreased incidence of GVHD is offset by an increased incidence of leukemic relapse, as well as failure of engraftment (688,689). Rather than depleting donor T cells in this situation, the goal should be to separate the graft-versus-leukemia (GVL) response from the graft-versuss-normal host tissue response.

Recently, several new approaches for inhibiting GVHD have been attempted. Some of these make use of similar immunosuppressive regimens to those being evaluated in solid organ transplant recipients, e.g., costimulatory blockade, alone (690,691) or in combination with adhesion molecule blockade (692). Peptides containing the CDR3 portion of the mouse CD4 molecule have been shown to be capable of inhibiting both GVHD (693) and resistance to allogeneic marrow engraftment (694). Treatment with anti-CD40L mAb also has been shown to inhibit GVHD and to promote allogeneic marrow engraftment (655,691). Because these approaches might be expected to block donor antihost responses nonspecifically, including those that eliminate residual leukemia in the host, it seems quite likely that they will also impair GVL responses. Alternative approaches involve immunostimulatory cytokines such as IL-2, IFN-γ, or IL-12, all of which, paradoxically, inhibit GVHD in mouse models (695–697). These cytokines are of interest because they could potentially mediate GVL effects while inhibiting GVHD. Indeed, these cytokines have been shown to preserve or enhance GVL effects while GVHD is inhibited (698–700). The use of nondepleting anti-CD3 F(ab)'2 fragments *in vivo* has also shown promise in a mouse model for the ability to maintain GVL effects while attenuating GVHD (701). Recently, rodent (702) and humanized (703) anti-CD25 mAbs have been evaluated for the treatment of acute GVHD, with only modest efficacy. In addition, mAbs to proinflammatory cytokines such as TNF-α (704) and an IL-1 receptor antagonist (705) have shown some efficacy in GVHD prophylaxis or treatment in initial clinical trials.

Another approach to separating the GVL potential from the GVHD-inducing capacity of MHC-directed alloreactivity is to separate the hematopoietic cell transplant and the administration of donor T cells in time, so that the T cells are given after some host recovery from the initial conditioning regimen has occurred (706–708). Apparently, host recovery from the injury associated with conditioning or the recovery of regulatory cell populations confers greater resistance to the induction of GVHD at late time points post-BMT compared with that which can be induced by T cells given at the time of conditioning. This resistance may explain the unexpectedly manageable level of GVHD that has been observed in patients who receive delayed donor leukocyte infusions to treat chronic myelogenous leukemia that has relapsed late after allogeneic BMT (709–711).

Several groups have investigated the possibility that $CD4^+$ or $CD8^+$ T-cell subsets could be identified that promote engraftment and GVL effects but not cause GVHD (712–714). Clinical studies involving selective CD8 depletion in HLA-identical sibling transplantation have shown a higher incidence of engraftment failure than is observed for unmanipulated BMT, but the rate was lower than that observed for pan-T cell–depleted transplants, and evidence for an antileukemic effect was obtained (715).

Another approach to separating the beneficial from the harmful effects of allogeneic T cells involves the *in vitro* expansion and cloning of donor T cells that selectively recognize leukemia-associated antigens but that do not recognize nonmalignant recipient cells. The existence of such CTL clones has been reported in mice (716) and humans (717–719). Such T cells are probably rare, and in order to separate them from GVHD-producing T cells, prolonged *in vitro* selection, cloning, and expansion is likely to be required. Such prolonged cultures may be impractical for use in the setting of BMT, in which leukemia-reactive cells must eliminate exponentially expanding leukemic cells. One class of leukemia-specific antigens that has recently stimulated intense research is the idiotypic determinants associated with unique Ig receptors and T-cell receptors on the surface of B- and T-cell malignancies, respectively, which may be particularly effective at immunizing when presented by professional APCs such as dendritic cells (720–722).

Xenogeneic Transplantation

The increasing shortage of cadaver donor organs has evoked a worldwide resurgence of interest in xenotransplantation, i.e., the replacement of human organs or tissues with those from a donor of a different species. Routine clinical application of this therapeutic modality is still in the future. However, recent progress, which will be reviewed briefly here, offers cause for optimism.

Concordant Versus Discordant Xenotransplantation

Xenotransplants have been classified into two groups, concordant and discordant, on the basis of phylogenetic distance between the species combination, speed of the rejection, and levels of detectable preformed antibodies. Animals that are evolutionarily closely related and do not have preformed natural antibodies specific for each other are called concordant, whereas animals that belong to evolutionarily distant species and reject organs in a hyperacute manner are termed discordant. There are, of course, many gradations between these extremes, and there are also a variety of known exceptions to the rule, making this nomenclature less than ideal.

Choice of Donor Species for Clinical Xenotransplantation

From a phylogenetic viewpoint, nonhuman primates would undoubtedly be the most similar to allotransplants immunologically. However, due to considerations of size, availability, and likelihood of transmission of infectious disease, most investigators have decided against the use of primates as a future source of xenogeneic organs. Instead, the discordant species, swine, has been chosen by many as the most suitable xenograft donor. The pig has essentially unlimited availability, as well as favorable breeding characteristics and the similarity of many of its organ systems to those of humans. Partially inbred miniature swine are a particularly attractive choice, because of their size (adult weights of approximately 120 kg), physiology (also similar to humans for many organ systems), and breeding characteristics, which have permitted inbreeding and genetic manipulation (723).

Mechanisms of Xenograft Rejection

Xenografts are subject to all four of the rejection mechanisms described earlier in this chapter and give rise to more powerful immune responses than allografts, probably for each type of rejection. There are two fundamental reasons for this finding. First,

xenografts offer more foreign antigens as targets for an immune response. Second, there are frequently molecular incompatibilities between members of different species that prevent the normal function of receptor–ligand interactions. Because in many cases the occurrence of homologous restriction for receptor–ligand pairs has been found to impair the regulatory processes that normally control immune and inflammatory responses, the result is that rejection mechanisms that may be relatively weak in allogeneic combinations become explosive after xenogeneic transplantation.

The well-recognized susceptibility of xenografts toward hyperacute rejection demonstrates both of these fundamental problems. As described earlier, pigs express an endothelial carbohydrate determinant, gal α(1,3) gal, that is not expressed by humans (724). As a result of this additional foreign antigen, pigs, in effect, express a new blood group antigen relative to all human recipients; thus, their organs are subject to hyperacute rejection, initiated by the binding of preformed natural antibodies. However, the hyperacute rejection that occurs with pig-to-primate transplantation is more vigorous than in the case of allogeneic blood group disparities. At least in part, this is because the complement regulatory proteins expressed by pig endothelium are less efficient in controlling human complement activation than are the human regulatory proteins expressed by human organs (180). Thus, these molecular incompatibilities contribute to the increased intensity of the hyperacute rejection mechanism.

Similarly, the factors responsible for accelerated graft rejection are more prominent in xenogeneic than in allogeneic transplantation. The rapid induction of an antibody response against xenografts probably reflects the expression of additional foreign antigens and the existence of preformed antibodies to these antigens, although at levels too low to initiate hyperacute rejection (142–144). In addition, the process of accelerated rejection is magnified considerably in xenografts by the failure of such regulatory molecules as tissue factor protein inhibitor to function effectively with human factor Xa, thus increasing the tendency toward intravascular thrombosis (200). The likely participation of NK cells in this form of xenograft rejection probably also reflects both the presence of additional antigens and the importance of molecular incompatibilities in xenotransplantation because novel carbohydrate determinants on pig endothelium may contribute to NK cell activation, and the molecular incompatibilities between human NK inhibitory receptors and swine class I molecules allows this activation to proceed without inhibition (725).

The available evidence also suggests that cell-mediated immune responses to xenografts are more powerful than to allografts (468). Initially, there was some uncertainty about this point because cell-mediated immune responses to xenogeneic stimulating cells were first studied using mouse T cells, for which molecular incompatibilities between species are actually responsible for a weaker direct recognition of xenogeneic than allogeneic stimulators *in vitro* (726). In this case, the incompatibilities turned out to involve the accessory molecules that are required for T-cell activation rather than molecules that inhibit a T-cell response. Thus, it seemed that cell-mediated rejection *in vivo* also might be weak. However, cell-mediated xenograft rejection, even by mice, has consistently been found to be extremely powerful, apparently initiated by $CD4^+$ T cells responding to the many additional antigenic peptides through the indirect pathway (727).

More recently, attention has been directed at investigation of the clinically relevant human–antipig cellular response. In contrast to the murine studies, direct responses by human T cells to pig stimulators can easily be measured *in vitro* (728,729). In addition, the cell-mediated reaction *in vitro* has been found to include a significant contribution by NK cells that can lyse pig targets (728,730). Thus, in the human–antipig combination, an important molecular incompatibility is the failure of human NK inhibitory receptors to interact with pig class I molecules. Numerous other molecular interactions that might be important in human–antipig T-cell responses have been examined (Table 6). With the exception of an apparently lower affinity of human CD8 for its binding site on pig class I molecules (which diminishes the direct human $CD8^+$ helper response to pig stimulators, and the failure of human IFN-γ to stimulate pig endothelium), the other molecular interactions appear to be at least partially functional (731).

The results of these studies have suggested that human–antipig T-cell responses are likely to be as great or greater than those in allogeneic combinations. Some investigators have identified a stronger indirect response by human T cells to pig stimulators than to allogeneic stimulators (732). If murine studies are correct in suggesting that the true basis of the strength of cell-mediated xenograft rejection lies in the strength of the indirect sensitization, this stronger indirect proliferation may indicate that human cell-mediated rejection of pig organs, both acutely and chronically, will indeed be more difficult to control than for allografts. Presumably, the source of this stronger indirect response lies partly in the larger number of foreign antigenic peptides generated by the disparate proteins of xenogeneic donors.

Therapeutic Strategies for Xenotransplantation

Three main strategies have been pursued to achieve long-term survival of xenogeneic transplants. The first has been to seek nonspecific immunosuppressive drugs that might prove especially effective for xenotransplantation. This approach was used successfully to achieve the excellent survival of allografts in current clinical practice. However, each new drug that has contributed to better outcomes for allografts has been tested experimentally for xenografts, and none has so far proven to be the magic bullet that might make xenografting possible. Based on our scientific understanding of the immunologic barriers to xenotransplantation, it is unlikely that any such drug exists. Furthermore, the heightened

TABLE 6. *Molecular interactions between human and pig*

Molecular interactions that are at least partially functional	
Human	Pig
TCR	SLA
CD4	SLA class II
CD8	SLA class I (±)
CD2	LFA-3 (±)
LFA-1	ICAM
CD28	B7
VLA-4	VCAM
Fas	FasL
Molecular interactions that are significantly impaired	
Human	Pig
NH KIRs	SLA class I
CD8	SLA class I
IFN-γ	IFN-γ R

immune response to xenografts compared with allografts suggests that larger amounts of exogenous immunosuppression would be required to achieve xenograft survival comparable with that of allografts. Given the narrow therapeutic window that already exists in clinical transplantation, most investigators believe that more than just immunosuppressive drugs will be needed to accomplish widespread clinical application of xenogeneic transplantation.

The second therapeutic approach has been to use genetic engineering of donor animals to lessen the immunologic barriers to xenografts. Because the two features that distinguish xenografts from allografts are the larger number of antigens and the molecular incompatibilities between species, these genetic modifications have been aimed primarily at correcting these two disadvantages of xenografts (Fig. 15). In mice, the technology of homologous recombination has made it possible to eliminate the expression of some of the deleterious genes. For example, knock-out mice have been generated that do not express the galactosyltransferase that is responsible for generating the α (1,3) gal determinant (733,734). However, in larger animals, including pigs, this technology is not currently available, limiting genetic modification of these animals to the insertion of transgenes. The most exciting application of this approach so far has been the creation of so-called hDAF-pigs, which are animals that express the human gene for the DAF complement regulatory protein (735,736). Organs from these animals appear to be significantly less susceptible to hyperacute rejection than are those from wild-type pigs. Numerous other potential transgenes are currently being examined experimentally, including genes for other complement regulatory proteins, genes encoding the human fucosyltransferase that produces the human blood group O determinant from the same substrate used by the pig galactosyltransferase, and genes encoding a glycosidase that might remove the α gal determinant. Almost certainly still other transgenes will be tested that might alter the process of accelerated rejection (perhaps by restoring normal thromboregulation or by promoting accommodation) or that might affect the cell-mediated mechanisms of graft destruction (perhaps by expressing human class I analogs to inhibit NK cells or by expressing downregulating molecules for T cells, such as Fas ligand).

The third strategy to achieve xenotransplantation is to eliminate the immunologic disadvantage of animal donors by inducing tolerance to the donor antigens. Potential applications of this strategy have been described earlier in this chapter. A key feature of this approach is that the techniques to achieve peripheral tolerance induction, which have appeared promising in allogeneic combinations, have generally been less successful even in closely related xenogeneic combinations. Deletional tolerance strategies have been the only ones that have achieved truly long-term xenograft survival using the stringent test of skin graft transplantation (153,154).

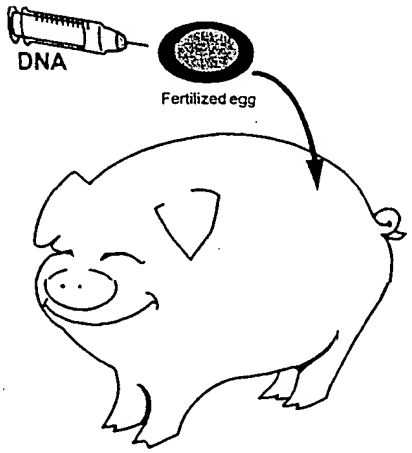
There is, of course, no reason to suggest that these three strategies are mutually exclusive, and many investigators believe that some combination of all three will be needed to accomplish xenogeneic organ transplantation on a large scale.

Nonimmunologic Barriers to Xenotransplantation

In addition to the immunologic mechanisms that prevent successful xenografting, there are two other potentially important obstacles to clinical application. First, the same kinds of molecular incompatibilities between species that alter immune responses may cause physiologic dysfunction of xenogeneic organs. For example, it appears that erythropoietin produced by pig kidneys does not function well in primates, causing progressive anemia in recipients with long-surviving pig kidney transplants (737). Presumably, there are many other such examples that will become apparent when long-term survival of metabolically complex organs, such as the liver, can be accomplished using discordant donors. On the other hand, there are also many examples where physiologic function remains intact across species differences, such as the ability of pig insulin to regulate human glucose appropriately. In addition, future examples of physiologic dysfunction are likely to be correctable using transgenic technology. Thus, the physiologic dysfunction of xenogeneic organs is unlikely to be an insurmountable barrier to all forms of xenotransplantation, although it may impair the function of certain types of xenogeneic organs or tissues.

The other nonimmunologic barrier to xenotransplantation is the possibility that successful tissue transplantation may allow the cross-species transfer of infectious agents, potentially creating a public health hazard for society as a whole. This possibility has only recently gained significant attention, and the issue has become confused by enormous uncertainties about the true risks that are involved.

"Zoonosis" is a term that has been used for some time to describe the general process of cross-species infection. More recently, the term "xeno-zoonosis" has been developed to describe



- Complement inhibition
  - DAF
  - CD46
  - CD59
- Antibody binding
  - Galactosidase
  - Fucosyl transferase
- Growth factors
  - pIL-3, pSCF
  - Human GF receptors
- MHC genes
  - Class I (NK inhibition)

FIG. 15. Transgenic pigs as xenograft donors. Some of the genes that have been used or have been considered for use in making transgenic pigs for xenograft donor.

infection transmission that might occur as a result of xenotransplantation. It is important to acknowledge that from the point of view of the individual recipient, the risk of transmitting infection by xenotransplantation is likely to be less than by current clinical allotransplantation, both because of the natural resistance to cross-species transmission of infectious diseases and because of the much longer time available to screen prospective donors. It is also important to point out that the risk of infectious transmission is unlikely to come from known pathogens because if the agent is known, it is generally possible to screen for and eliminate its presence.

The major concern, therefore, regarding infections resulting from xenotransplantation is that endogenous retroviral sequences from donor cells might infect the recipient's cells, giving rise themselves, or after recombination with human endogenous retroviral sequences, to previously unrecognized pathogenic viruses. Such new viruses might prove hazardous to other human beings in addition to the xenograft recipient. Although it has seemed to some that cross-species transmission of retroviruses would probably have occurred already in nature if it was likely to happen, others have pointed out that the circumstances of xenotransplantation may create unique conditions favoring this event. In particular, the prolonged coexistence of cells from two different species in patients who are taking immunosuppressive drugs or who have been rendered tolerant to their donors may be especially permissive for cross-species transfer of endogenous retroviruses. This concern has increased as a result of *in vitro* studies showing that pig proviruses can infect human cells when cells from both species are cultured together (738). At this time, however, there is no evidence that such cross-species transfer after a pig-to-human transplant would generate a virus that would be infectious or pathogenic. Because the concern about infections from xenotransplantation involves fear of an event that has never been known to happen, it is impossible to assign an accurate level of risk. Presumably it is low, but on the other hand, the consequences of the unlikely event might prove to be large for the many members of the human species who would receive no direct benefit from xenotransplantation. At this time, therefore, health agencies and members of the transplant community are attempting to design rational approaches for identifying the true risks of xenotransplantation and detecting untoward events rapidly, while at the same time allowing further progress in this potentially enormously important field of transplantation.

Clinical Progress in Xenotransplantation

Given the serious obstacles to successful xenotransplantation described above, it may be surprising that clinical trials using animal donors for humans have been, and are being, conducted. Many of the early efforts took place in the 1960s and involved organ transplants from nonhuman primates (183). One of the patients survived for nine months with normal renal function provided by the kidney of a chimpanzee (739). The most recent clinical trials have involved fetal pig cells transplanted into the brains of patients with Parkinson's or Huntington's diseases. The documented survival of pig tissue 8 months after the transplant in a patient taking only moderate doses of cyclosporine suggests that the blood–brain barrier provides a degree of immunoprotection (740). These studies also suggest that cellular xenotransplantation may be achieved more easily and thus may be performed sooner than solid organ transplants, especially because free cellular transplants lack the vascular endothelium that is the target for both hyperacute and accelerated rejection.

SOME IMMUNOLOGIC ISSUES IN CLINICAL TRANSPLANTATION

The Effect of Antigen Matching on Graft Survival

Clinical Evidence

Transplantation antigens are defined by their ability to cause graft rejection, and in the absence of transplantation antigen disparities, graft rejection does not occur. Thus, there can be no argument with the statement that antigen matching improves graft survival. Contrary to this simple conclusion, however, the importance of antigen matching is one of the more controversial issues in clinical transplantation. The debate is frequently confused by failure to focus on the relevant quantitative issue of whether improved, but incomplete, antigen matching influences the outcome of organ transplantation sufficiently under current clinical circumstances to warrant its logistic difficulties.

The evidence from transplantation of kidneys using living-related donors provides a clinical demonstration of the importance of antigen matching in subsequent graft survival. Two siblings may share all of their HLA antigens (25% likelihood), half of their HLA antigens (50% likelihood), or none of their HLA antigens (25% likelihood). Identical twins share all of their transplantation antigens, but siblings are generally matched for only about half of the minor antigens, which distinguish their parents, even if they are HLA identical. Table 7 shows one institution's survival rates for kidney grafts after 1 year for HLA-identical and one-haplotype–matched living-related donors. Similar differences have been reported in the UCLA kidney transplant registry (741,742). The data in Table 7 indicate that some graft loss occurs even for HLA-identical siblings (indicating the role of minor histocompatibility antigens even in the face of immunosuppression) and that the outcome of kidney transplantation is better when there is complete HLA matching compared with only partial matching. These data support the basic concept that antigen matching matters, and for related donors, MHC antigen matching is widely agreed to be advantageous.

In the absence of a living-related donor, transplantation is performed with organs from unrelated donors, usually from cadaveric sources. Because of the extensive polymorphism of MHC antigens, unrelated donors selected in a random fashion would not be expected to share many HLA antigens with the recipient. Similarly, there would only be sporadic matching of the minor histocompatibility antigens. Correspondingly, the survival of organs from cadaveric donors has generally been poorer than that of HLA-identical or one-haplotype–matched siblings as shown by the data in

TABLE 7. *One-year kidney graft function*

	1996	1986	1976
HLA-identical grafts (living-related)	100%	100%	90%
One-haplotype matched grafts (living-related)	94%	92%	78%
Cadaver–Donor Grafts	86%	83%	58%

Data from the Transplantation Unit, Massachusetts General Hospital, Boston.

Table 7. The clinical issue under these circumstances is whether or not the nonrandom distribution of organs to achieve a larger number of matched antigens would achieve better results.

Figure 16 shows the effect of antigen matching for cadaver donor renal transplantation according to the data in the UCLA kidney transplant registry (741). The results suggest that for cadaver donor transplantation, only near-perfect MHC antigen matching leads to detectable benefit, and that the benefit is probably less than 10% in 1-year allograft survival. Not all studies of the effect of antigen matching in cadaver donor transplantation have shown the same benefit (743–748). Many factors probably affect our ability to measure the influence of antigen matching, including, for example, the finding that the success of transplantation, using poorly matched grafts, has varied considerably in different studies. Centers with poor graft survival might be expected to see greater benefit of antigen matching than centers that achieve high rates of success even under less favorable circumstances. Therefore, although antigen matching matters in the biology of transplantation, it probably matters little in modern clinical medicine.

Over the years the controversy about the impact of antigen matching has focused on several specific issues. Some investigators have suggested that matching for particular alleles of the HLA antigens is especially important, and others have suggested that matching for some loci is more important than for others (749,750). More recently, the issue has been further refined to include the question of whether matching for class II antigens might be more important than matching for class I antigens. There are clinical data suggesting that class II antigen matching is of particular benefit to the outcome of transplantation (751–753). Furthermore, it has recently become possible to perform class II typing with more precise molecular techniques than the serologic methods used in the past. Although the use of this technology could potentially increase the survival benefits associated with class II antigen matching, it would also reduce the likelihood that a matched donor could be found by these more stringent criteria and might increase the time required to perform HLA typing. Thus, it remains unclear that the distribution of organs to achieve such matching is worth the incumbent effort, expense, and increased ischemic time, which also may affect outcome.

Experimental Evidence

A particularly useful large animal model has been developed to test experimentally the importance of antigen matching. Over the past 20 years, three herds of partially inbred miniature swine have been developed for studies of transplantation biology. Each herd has been bred to homozygosity for a different allele at the MHC (termed SLA in swine) (40). Subsequent breeding has been intentionally randomized within herds in order to maintain a variety of segregating minor histocompatibility loci. Transplants among these animals thus resemble the situation within human families, i.e., HLA identical versus nonidentical siblings.

Studies of skin and renal allografts between these animals produced the results shown in Table 8. The difference observed for skin graft survival between SLA matched and mismatched animals was modest. Matching had a much more profound effect on kidney graft survival. One-third of the grafts between SLA-matched animals survived indefinitely without immunosuppression, despite the existence of multiple minor histoincompatibilities. The ability to reject renal allografts across minor differences was found to depend on an Ir gene, inherited in an autosomal-dominant fashion and not linked to the MHC (494).

Intra-MHC recombinants between several NIH minipig haplotypes have been identified, making it possible to examine the relative importance of class I versus class II matching on renal allograft survival in these large animals (40). The survival of renal allografts with class I–only differences and with class II–only differences is shown in Table 8. As in the mouse, both class I and class II differences appeared sufficient to cause prompt skin graft rejection. However, for kidney allografts, class II matching was of particular importance in determining the outcome. In fact, the results for minor plus class I differences were indistinguishable from those for minor histocompatibility antigen differences alone.

These experimental data were obtained without the exogenous immunosuppression always administered in clinical studies. They demonstrate the biologic principle that antigen matching is important to graft survival and further indicate that class II antigen matching is likely to be particularly important. No experimental system is likely to settle the empirical issue in clinical medicine,

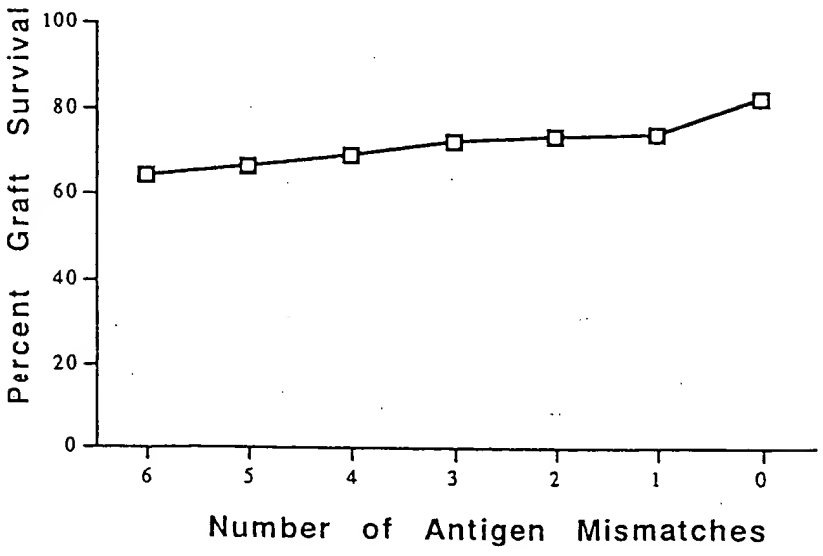


FIG. 16. Effect of antigen matching on graft survival. Three-year graft survival based on number of antigen mismatches.

TABLE 8. *Graft survival and antigen matching in minipigs without immunosuppression*

	Mismatch			
Graft	Minors only	Major and minors	Class I[a]	Class II[a]
Skin	11.8 ± 0.9	7.0 ± 0.4	10.8 ± 2.3	7.8 ± 1.0
Kidney	30.0 ± 15.0 (2/3) >120 (1/3)	12 ± 1.9 12.0	19.5 ± 6.8 (2/3) >120 (1/3)	21.8 ± 10.4

[a]Single haplotype mismatch
Survival time is shown in days.
Data from reference (40).

however, of how much benefit will be obtained under the conditions of current practice.

The Cross-Match

There are several means of detecting preexisting antibodies in the serum of potential recipients that have specificity for donor antigens. First, it is necessary to determine blood type because clinical transplantation across blood group barriers is never knowingly attempted for those organs susceptible to hyperacute rejection. One exception is the ability to transplant organs from donors of the A2 blood group to recipients of other blood groups (754). Secondly, immediate pretransplant sera from prospective recipients are tested against lymphocytes of potential donors. This test is called a cross-match, and it is not the same as antigen matching discussed above. An individual can have a negative cross-match (meaning that he or she does not have antibodies reactive with donor antigens) but still be completely unmatched with respect to HLA antigens. On the other hand, matching for some HLA antigens may improve the chances of obtaining a negative cross-match for prospective recipients who have developed antibodies reactive with many foreign HLA antigens. The cross-match is generally performed by a two-step antibody-mediated complement-dependent cytotoxicity assay. In many centers, the test is augmented by the intermediate addition of antihuman immunoglobulin to increase the sensitivity for detecting lysis (184,755). More recently, flow cytometry has been used to detect preexisting recipient antibodies with still additional sensitivity, although the data from flow cytometric analysis may be too sensitive to be clinically applicable (756–758).

The Sensitized Candidate for Organ Transplantation

Because kidneys and many other vascularized organs cannot currently be transplanted into recipients with preexisting antibodies, the clinical goal is to avoid the formation of antibodies reactive with donor antigens or to find organs that do not express the particular HLA antigens against which the recipient has been sensitized. Except for blood group antibodies, recipient sensitization to transplantation antigens always occurs by prior exposure to allogeneic tissue. This may occur as a result of blood transfusion, as a result of previous organ transplantation, or, in women, by exposure to paternal antigens during or just after pregnancy. The degree of sensitization of a potential kidney recipient is measured regularly by testing sera on a panel of lymphocytes selected from individuals who collectively express a broad representation of the HLA antigens. Transplantation candidates whose sera react with a high percentage of the cells in this panel (panel reactive antibody) are said to be "highly sensitized." The term may be confusing to immunologists because in the clinical setting it refers only to B-cell sensitization and does not necessarily imply sensitization of cell-mediated effector mechanisms. Highly sensitized candidates may wait years to receive a kidney transplant, and some may never receive one.

If a recipient has detectable antibodies to HLA antigens, they cannot receive an organ bearing these HLA antigens. Prior screening of potential recipients against the panel of HLA antigens can predict some of the determinants against which antibodies already exist. Most highly sensitized individuals actually produce antibodies of relatively limited heterogeneity that are reactive with public epitopes of HLA antigens (759). Thus, the HLA phenotype of unsuitable donors for any given recipient can be predicted with some precision. In this case, HLA tissue typing has value in identifying kidneys that may have a negative cross-match for highly sensitized individuals.

The level of sensitization manifested by transplantation candidates fluctuates over time. As a result, it is possible for recipients to have a negative cross-match with a donor's cells using recently obtained serum, but a positive cross-match using previously collected sera. Transplantation in the face of this historical positive cross-match has been performed successfully (760).

Obtaining cross-match–negative donors by locating well-matched organs or by waiting for a decline in the level of sensitization represent the primary solutions currently available for sensitized patients. Despite numerous trials, no widespread protocol for the active treatment of sensitized individuals to remove antibody has been adopted.

The Diagnosis of Rejection

In clinical organ transplantation, the most obvious manifestation of the rejection process is diminished function of the transplanted organ. Other causes of graft dysfunction exist, however, and it is obviously important to confirm the immunologic origin of the event before increasing immunosuppression. The clinical pattern of dysfunction often helps to suggest the diagnosis of rejection. However, no clinical sign can definitively diagnose rejection. It would be useful, therefore, to determine a means of identifying rejection episodes based on systemic manifestations of the immunologic mechanisms involved. Unfortunately there is not yet a well-established assay to measure rejection activity. Two approaches include the measurement of antidonor antibody production and the sequential measurement of cell-mediated responses to donor antigens. Antibody responses have frequently been documented following

graft rejection, but they tend to appear after rejection is complete. *In vitro* cell-mediated responses, both proliferative and cytotoxic, may or may not be present while a graft is in place and are not well correlated with clinical rejection episodes (761,762). Assays of humoral and cellular responses to donor antigens both suffer from the possibility that donor-specific elements of the response may be absorbed by the antigens of the graft, at least until the late stages of rejection. Furthermore, the existence of tissue-specific peptides may allow T-cell responses to occur *in vivo* that cannot be measured *in vitro* when donor lymphocytes are used as stimulators.

The standard procedure in the diagnosis of allograft rejection has always been the biopsy of the transplanted organ itself. Pathologists have been able to identify the abnormal lymphocytic infiltrate within grafts, to grade the intensity of the infiltrate, and, for some organs, to describe histologic findings characterizing the effects of immunologic injury (763–769). Some pathologic changes, including a lymphocytic infiltrate of the vascular wall, seem to be well correlated with rejection activity (769). In addition, pathologic changes suggesting nonimmunologic causes of renal dysfunction may be helpful in patient management.

Despite the widespread reliance on the biopsy to define episodes of rejection, however, differentiation of rejection from its absence is often difficult, particularly when cyclosporine immunosuppression has been used. Because most clinical allograft biopsies are performed when the organ is not functioning well, and only after mechanical causes of dysfunction have been excluded, most organs that are examined via biopsy, by selection, are undergoing rejection. Therefore, inability to detect nonrejection events in a few cases pathologically will still leave an excellent correlation between the diagnosis of rejection and the response to therapy. When routine biopsies of transplanted organs have been done, regardless of organ dysfunction, they have revealed a poor correlation between histologic findings and clinical evidence of rejection (770,771). Experimental studies of skin grafts, as discussed above, have found that the degree of lymphocytic infiltrate in an allograft correlated better with the nature of the antigenic disparity rather than with the intensity of the rejection process (285). Furthermore, several experimental models of tolerance induction have shown intense lymphocytic infiltrates in organs that go on to survive indefinitely in recipients who develop tolerance to the donor antigens (772). These studies suggest that the amount of lymphocytic infiltrate detected pathologically may not be helpful in diagnosing rejection episodes and determining the need for treatment.

How Much Immunosuppression Is Enough?

Although the majority of transplant recipients respond immunologically to their new organ despite immunosuppression, some patients seem never to generate any rejection activity and maintain their transplanted organ with small doses of immunosuppressive drugs. Indeed, a few patients have been known to stop all of their medications but have kept their transplant for years without rejection. On the other hand, some patients seem to require and tolerate high doses of exogenous immunosuppression, whereas others seem to be severely immunocompromised by low doses of these drugs. These observations make it clear that the amount of immunosuppression that is required or that is safe is not the same for every individual or for all grafts. Unfortunately there is no well-established assay to determine the amount of immunosuppression an individual requires and can safely tolerate for their particular transplant.

Recently, a biologic determination of immunocompetence for patients on cyclosporine has been reported. Lymphocytes from transplant recipients were tested for their capacity to generate $CD4^+$ direct helper responses, $CD8^+$ direct helper responses, and $CD4^+$ indirect helper responses to foreign antigens. Those patients whose direct responses were intact appeared to experience more rejection than those who only maintained their indirect responses (36,773). Refinement of assays such as this, especially to incorporate donor-specific responses, would help enable tailoring of immunosuppressive therapy for the individual patient.

CONCLUSION

The great danger in any textbook chapter is that the need to summarize what we think is known will obscure the much greater amount still left to be learned. In recent years enormous progress has been made in the study of the major histocompatibility antigens; yet we still know too little about the products of the numerous other histocompatibility loci that encode the minor antigens. Recently we have gained important insight into the role of APCs in T-cell sensitization; but we still have not explored adequately the role that indirect presentation of alloantigens plays in graft rejection. During the past two decades we have learned much about the generation and function of CTLs and about their likely role in some mechanisms of graft rejection; however, the suspicion is strong that noncytotoxic mechanisms of rejection probably also exist, involving a crucial role for cytokines. Finally, this chapter has outlined several techniques for the generation of immunologic tolerance to alloantigens in experimental systems; yet it is still not possible to accomplish routine organ transplantation in human beings without the use of nonspecific immunosuppression. It is, of course, the great fascination of transplantation immunology that new insights into these and other basic immunologic issues will likely have such important consequences in clinical transplantation.

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